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(54) Title: METHODS OF TRANSCRIPTIONALLY MODULATING EXPRESSION OF ONCOGENES AND TUMOR SUPPRESSOR GENES

(57) Abstract

The invention provides a method for directly effecting transcription of an oncogene or tumor suppressor gene; a method for testing compounds for directly effecting transcription by measuring the product polypeptide produced; and a method for testing compounds for directly effecting transcription by measuring the mRNA produced.

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METHODS OF TRANSCRIPTIONALLY MODULATING EXPRESSION OF ONCOGENES AND TUMOR SUPPRESSOR GENES

This application is a continuation-in-part of U.S. Serial No. 644,293, filed January 18, 1991, the contents of which are hereby incorporated by reference into the present application.

Background of the Invention

Throughout this application, various publications are referenced by Arabic numerals within parentheses. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

The genomes of higher eucaryotic cells contain specific genes that, when activated by any one of several mechanisms, are capable of transforming normal cells to the malignant state. These genes, termed oncogenes, can be loosely defined as genes whose abnormal expression or altered gene product directly contributes to the production of the malignant phenotype. More than 50 such oncogenes have been identified thus far, and current estimates suggest that as many as 200 may be found to exist.

Oncogenes derive from normal cellular progenitors, called proto-oncogenes, many of which code for proteins which

are involved in cell divisi n or differentiation. Proto-oncogene encoded products include growth factor-like molecules (int-2), receptors with tyrosine kinase activity (c-erbB2), nuclear proteins (c-myc), membrane bound proteins with GTPase activity (ras), transcription factors (c-jun) and hormone receptors (c-erbA1)(58). The generation of oncogenes from their non-transforming homologues can occur via several different mechanisms; (a) retroviral transduction of proto-oncogenes from a donor cell to host cell during viral replication, placing the donor's proto-oncogene under control of the strong retroviral promoter and resulting in the constitutive over production of a normal protein or in a mutated activation of the protein as a consequence of the transduction event. (b) gene amplification, leading to an increased concentration of the proto-oncogene product (c) binds to DNA or RNA, or chromosomal translocation, resulting in either a novel fusion protein with increased biochemical activity or else overproduction of the translocated cellular gene product by virtue of its placement in proximity to another cellular gene's strong promoter (d) mutation within a proto-oncogene's coding region resulting in a protein product with altered biochemical function(s).

Although a relatively large number of oncogenes with potential transforming capabilities exist, the majority have not been implicated in human malignancies. Only about 10 oncogenes have been clearly demonstrated to be involved in human disease.

Recently, a set of cellular genes different from ncogen s, namely tumor suppress r g nes or anti-oncogenes, have been implicat d in tumorigenesis. Anti-oncogenes have be n defin d as g n s whos repr ssion,

inactivation, dysfunction r loss results in c ll transformation. Tumor suppressor genes has been defined experim ntally as g nes whose introduction, activation or expression results in the inhibition or suppression of the tumorigenic phenotype. Clearly these definitions are not mutually exclusive, anti-oncogenes can function as tumor suppressor genes and vice-versa. The biochemical function(s) of anti-oncogene products remain to be elucidated; however, two anti-oncogene products, Rb, the protein product of the cellular retinoblastoma gene, and p53, have been shown to bind transforming viral proteins. Loss or inactivation of anti-oncogenes (by allelic deletion or mutational alteration) may therefore be involved in tumorigenesis by their failure to interact with and suppress the activity of transforming oncogenes. In several cases a direct role of tumor suppressor in control of the cell cycle has been implied.

As stated previously, only a limited number of oncogene and anti-oncogenes have been shown to be present in human cancers. Within this group, an even smaller number are thought to play a critical role in a large percentage of specific cancers or multiple type of neoplasias. The following is a brief description of those genes which belong to this latter category.

A. ras: in mammalian species, the ras family of proto-oncogenes consists of three closely related members, termed H, K, and N-ras. Each codes for a 21 kd protein (p21) with GTP binding and hydrolysis activities. The most common mechanism of activation of the ras oncogene is a signal point mutation, frequently occurring in the protein's twelfth codon, which results in a decreased GTPase activity and a c ncomitant acquisition f transforming potential in

experim ntal systems. Furtherm re, ras mutations ar found in a large perc nt of cancers, supporting the supposition that the mutated protein's altered biochemical function plays a critical role in neoplasias. Specifically, mutated K-ras has been found in adenocarcinoma of the lung, colon and pancreas, occurring in 30%, 50% and greater than 70% of those tumors, respectively (1,2)

K-ras mutations have also been reported in over 20% of melanoma tumor samples recently examined (3). Between 50 and 70% of the different tumor types are refractory to current methods of therapy and eventually prove fatal.

In addition to the preponderance of mutated ras protein in human cancer, a number of reports suggests a link between overexpression of the normal ras gene and neoplastic transformation (4,5,6). Regardless of the mechanism of activation, the ras oncogene is the most implicated of all oncogenes in its wide range and high percentage that is found in a variety of human neoplasias.

B. erbB2 (neu): The erbB2 gene encodes a 185 Kd transmembrane protein-tyrosine kinase which closely resembles the EGF receptor. Amplification of the erbB2 gene has been found to occur in 30% of breast cancer (7). Amplification is found in both early and late stages of disease, and is maintained in the metastases. Furthermore, there is a direct correlation between gene amplification and overexpr ssion of the erbB2 protein, although numerous examples of tumors which overexpress

protein in the absence of gene amplification also exist. Overexpression of the erbB2 protein also occurs in 30% of varian cancers. In both tumor types, erbB2 overexpression correlates with poor patient prognosis, with a shorter time to relapse as well as a decreased overall survival rate.

In experimental systems, overexpression of the normal human erbB2 gene in murine fibroblasts to levels similar to those in human breast and ovarian cancers have been shown to induce cell transformation (8,9). In addition, erbB2 overexpression in the human MCF-7 breast cancer line allows these cells to grow as tumors in nude mice. Furthermore, monoclonal antibodies to human erbB2 have specific anti-proliferative effects in vitro of human breast tumor cells which overexpress erbB2 (10). These and other data strongly suggest that the erbB2 gene plays an important role in the onset and progression of breast and other cancers.

C. p53: The first host protein shown to bind specifically to the product of a DNA tumor virus oncogene (SV40 large T-antigen) was p53 (11,12).

Recent studies examining the genetic alterations which occur during the development of colorectal tumors have indicated a common mutation to be deletions of the short arm of chromosome 17, the region where the p53 gene is located. Chromosome 17p deletions are often late events associated with the transition from the benign (adenomatous) to the malignant (carcinomatous) state (13). In a vast majority of colorectal carcinomas examined, allelic deletions of 17p were found to occur. The remaining

p53 allele was shown to contain point mutations suggesting that p53 gene mutati ns may be involved in colorectal neoplasias, perhaps through inactivation of a tumor suppressor function of the wild type p53 gene (14). Allelic loss of p53 (chromosome 17p) has also been observed in a large number of breast and lung tumors analyzed. In a study involving a limited number of hepatocellular carcinoma-derived cell lines, six of seven showed p53 abnormalities (15) suggesting a general mechanism of p53 inactivation in several different human neoplasias (16,17,18) Recently specific mutations in p53 have been found in a large percentage of cases of malignant melanoma.

The specific cellular function of p53 has not yet been determined, although several lines of evidence suggest a role in the regulation of DNA replication (19). Loss of one p53 allele followed by mutational inactivation of the remaining allele could therefore result in a mutated p53 protein incapable of carrying out its normal function, one proposed function being tumor suppressor activity. There is increasing evidence that mutated p53 may play an active role in transformation suggesting that p53 may act as either an oncogene or an anti-oncogene depending on whether it is mutant or wild-type.

Pharmaceuticals which increase or decrease the expression of oncogenes, anti-oncogenes or tumor suppressor genes will have important clinical applications for the treatment of various neoplasias. We describe herein a method for discovery of compounds which modulate the expression of these genes and describe the use of such compounds. The g neral appr ach is to scr en compound

librari s for substanc s which increas or decrease expression f oncogenes, anti-oncogen s, tumor suppressor gen s or proto-oncog n s.

The expression of a specific gene can be regulated at any step in the process of producing an active protein. Modulation of total protein activity may occur via transcriptional, transcript-processing, translational or post-translational mechanisms. Transcription may be modulated by altering the rate of transcriptional initiation or the progression of RNA polymerase (28). Transcript-processing may be influenced by circumstances such as the pattern of RNA splicing, the rate of mRNA transport to the cytoplasm or mRNA stability. This invention concerns the use of molecules which act by modulating the in vivo concentration of their target proteins via regulating gene transcription. The functional properties of these chemicals are distinct from previously described molecules which also affect gene transcription.

Researchers have documented the regulation of transcription in bacteria by low molecular weight chemicals (20, 21). Extracellular xenobiotics, amino acids and sugars have been reported to interact directly with an intracellular proteinaceous transcriptional activator or repressor to affect the transcription of specific genes.

Transcriptional regulation is sufficiently different between procaryotic and eucaryotic organisms so that a direct comparison cannot readily be made. For example, procaryotic cells lack a distinct membrane bound nuclear compartment. Furthermore the structure and organization of procaryotic DNA l ments resp nsible for initiation of

transcription differ markedly from thos of eucaryotic cells.

The eucaryotic transcriptional unit is much more complex than its procaryotic counterpart and consists of additional elements which are not commonly found in bacteria, including enhancers and other cis-acting DNA sequences (22, 23). Procaryotic transcription factors most commonly exhibit a "helix-turn-helix" motif in the DNA binding domain of the protein (24, 25). Eucaryotic transcriptional factors frequently contain a "zinc finger" (25, 26), "helix-loop-helix" or a "leucine zipper" (27) in addition to sometimes possessing the "helix-turn-helix" motif (28). Furthermore, several critical mechanisms at the post-transcriptional level such as RNA splicing and polyadenylation are not typically found in procaryotic systems (29, 30).

In higher eucaryotes, modulation of gene transcription in response to extracellular factors can be regulated in both a temporal and tissue specific manner (31). For example, extracellular factors can exert their effects by directly or indirectly activating or inhibiting tissue specific transcription factors (31, 32).

Modulators of transcription factors involved in direct regulation of gene expression have been described, and include those extracellular chemicals entering the cell passively and binding with high affinity to their receptor-transcription factors. This class of direct transcriptional modulators include steroid hormones and their analogs, thyroid hormones, retinoic acid, vitamin D_3 and its derivatives, and dioxins, a chemical family of polycyclic aromatic hydrocarbons (26, 33, 34).

Di xins ar molecules generally known to modulat transcription, however, dioxins bind to naturally-occurring receptors which respond normally to xenobiotic agents via transcriptionally activating the expression of cytochrome P450, part of an enzyme involved in detoxification. Similarly, plants also have naturally occurring receptors to xenobiotics to induce defense pathways. For example, the fungal pathogen Phytophthora megasperma induces an anti-fungal compound in soybeans. Such molecules which bind to the defined ligand binding domains of such naturally occurring receptors are not included on the scope of this invention.

The clinical use of steroid hormones, thyroid hormones, vitamin D_3 and their analogs demonstrates that agents which modulate gene transcription can be used for beneficial effects, although these agents can exhibit significant adverse side effects. Obviously, analogs of these agents could have similar clinical utility as their naturally occurring counterparts by binding to the same ligand binding domain of such receptors. These types of molecules do not fall within the scope of this invention because they function by binding to the ligand-binding domain of a receptor normally associated with a defined physiological effect.

Indirect transcriptional regulation involves one or more signal transduction mechanisms. This type of regulation typically involves interaction with a trans-membrane signal transducing protein, the protein being part of a multistep intracellular signaling pathway, the pathway ultimately modulating the activity of nuclear transcription factors. This class of indirect transcriptional modulators include polypeptide growth factors such as platelet-derived growth factor, epidermal

growth fact r, cyclic nucleotide analogs, and mitogenic tumor promoters such as PMA (35, 36, 37).

It is well documented that a large number of chemicals, both organic and inorganic, e.g. metal ions, can non-specifically modulate transcription. Most heavy metals modulate gene expression through receptors in a mechanism similar to that employed by dioxin, steroid hormones, vitamin D3 and retinoic acid.

Researchers have used nucleotide analogs in methods to non-specifically modulate transcription. The mechanism involves incorporating nucleotide analogs into nascent mRNA or non-specifically blocking mRNA synthesis. Similarly, researchers have used alkylating agents, e.g. cyclophosphamide, or intercalating agents, e.g. doxorubicin, to non-specifically inhibit transcription.

Moreover, chemical inhibitors of hydroxymethyl-glutaryl CoA reductase, e.g. lovastatin, are known to indirectly modulate transcription by increasing expression of hepatic low density lipoprotein receptors as a consequence of lowered cholesterol levels.

Signal effector type molecules such as cyclic AMP, diacylglycerol, and their analogs are known to non-specifically regulate transcription by acting as part of a multistep protein kinase cascade reaction. These signal effector type molecules bind to domains on proteins which are thus subject to normal physiological regulation by low molecular weight ligands (38, 39).

The specific use of sterol regulatory elements from the LDL receptor gene to control expression of a r porter gene has r cently be n documented in PCT/US88/10095. One

aspect of PCT/US88/10095 deals with the use of specific sterol regulatory elements coupled to a reporter as a means to screen for drugs capable of stimulating cells to synthesize the LDL receptor. PCT/US88/10095 describes neither the concept of simultaneously screening large numbers of chemicals against multiple target genes nor the existence of transcriptional modulators which (a) do not naturally occur in the cell, (b) specifically transcriptionally modulate expression of the gene encoding the oncogene or tumor suppressor gene product, and (c) binds to DNA or RNA, or bind to DNA or RNA or bind to a protein through a domain of such protein which is not a defined ligand binding domain of a nuclear, transcriptionally activating receptor which naturally occurs in the cell, the binding of a ligand to which ligand binding domain is normally associated with a defined physiological effect. The main focus of PCT/US88/10095 is the use of the sterol regulatory elements from the LDL receptor as a means to inhibit expression of toxic recombinant biologicals.

The use of molecules to specifically modulate transcription of oncogenes and tumor suppressor genes as described herein has not previously been reported and its use will bring surprise since available literature does not propose the use of a molecule, as described, in a method to specifically modulate transcription. Instead, the available literature has reported methods which define domains of transcriptional regulating elements of a gene.

Further, the practice of using a reporter gene to analyze nucleotide sequences which regulate transcription of a gene-of-interest is well documented. The demonstrated utility of a reporter gene is in its ability to define

domains of transcriptional regulatory elements of a gene-of-interest. Reporter g nes which expr ss proteins, e.g. luciferase, are widely utilized in such studi s. Luciferases expressed by the North American firefly, Photinus pyralis and the bacterium, Vibrio fischeri were first described as transcriptional reporters in 1985 (40, 41). Reporter genes have not been previously used to identify compounds which (a) do not naturally occur in the cell, (b) specifically transcriptionally modulate expression of the gene encoding the oncogene or the tumor suppressor gene product, and (c) binds to DNA or RNA, or bind to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

A method to define domains of transcriptional regulating elements of a gene-of-interest typically has also involved use of phorbol esters, cyclic nucleotide analogs, concanavalin A, or steroids, molecules which are commonly known as transcriptional modulators. However, available literature shows that researchers have not considered using a transcription screen to identify specific transcriptional modulators. Apparently, success would be unlikely in doing so, however, we have demonstrated herein that this is not the case.

There is utility in developing the method of transcriptional modulation of oncogenes and tumor suppressor genes by using such molecule as described herein. This method will allow the development of novel pharmaceuticals and circumvent many of the problems associated with the therapeutic use of recombinant biological factors where clinical the use of prot in

asp ct f PCT/US88/10095 deals with th use f specific st r l regulatory lements c upled to a rep rter as a m ans to screen for drugs capable f stimulating cells to synthesize the LDL receptor. PCT/US88/10095 describes neither the concept of simultaneously screening large numbers of chemicals against multiple target genes nor the existence of transcriptional modulators which (a) do not naturally occur in the cell, (b) specifically transcriptionally modulate expression of the gene encoding the oncogene or tumor suppressor gene product, and (c) binds to DNA or RNA, or bind to DNA or RNA or bind to a protein through a domain of such protein which is not a defined ligand binding domain of a nuclear, transcriptionally activating receptor which naturally occurs in the cell, the binding of a ligand to which ligand binding domain is normally associated with a defined physiological effect. The main focus of PCT/US88/10095 is the use of the sterol regulatory elements from the LDL receptor as a means to inhibit expression of toxic recombinant biologicals.

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There is utility in developing the method of transcriptional modulation of oncogenes and tumor suppressor genes by using such molecule as described herein. This method will allow the development of novel pharmaceuticals and circumvent many of the problems associated with the therapeutic use of recombinant biological factors where clinical the use f pr tein

factors is relevant.

Problems associated with the therapeutic use of recombinant biological factors include the technical difficulties of large scale protein purification, the high costs of protein production, the limited shelf-life of most proteins and in some cases a short biological half-life of the administered protein in the organism. Additionally, therapeutic delivery of proteins normally requires injection. The method described herein provides a means of upregulating the expression of proteins which are not readily amenable to administration as injectable biologicals.

Furthermore, molecules specifically regulating the activity of one member of a group of closely related proteins are difficult to identify. Bioactive molecules, structurally related at the protein level, may possess distinct regulatory elements at the DNA level which control their expression. Thus, molecules such as the chemical transcriptional modulators defined herein can provide a greater opportunity for specifically modulating the activity of structurally related proteins.

Finally, the molecules described herein may also serve to mimic normal physiological response mechanisms, typically involving the coordinated expression of one or more groups of functionally related genes. Therefore, determining whether a molecule can specifically transcriptionally modulate the expression of an oncogene or tumor suppressor gene and the ultimate clinical use of the molecule provides a therapeutic advantage over the use of single recombinant biologicals, or drugs which bind directly to the final target protein encoded by the gene- f-interest.

Summary of the Invention

The invention provides a method of directly transcriptionally modulating the expression of an oncogene or a tumor suppressor gene, the expression of which is associated with a defined physiological or pathological effect within a multicellular organism. This method comprises contacting a cell, which is capable of expressing the gene, with a molecule at a concentration effective to transcriptionally modulate expression of the gene and thereby affect the level of the oncogene or the tumor suppressor gene product(s) encoded by the gene which is expressed by the cell. In this method the molecule (a) does not naturally occur in the cell and (b) specifically transcriptionally modulates expression of the oncogene or the tumor suppressor gene, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

The invention further provides for a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of an oncogene or a tumor suppressor gene. This method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the oncogene or the tumor suppressor gene, (ii) a promoter of the oncogene or the tumor suppressor gene, and (iii) a DNA sequence encoding

a polypeptid other than the oncog n or the tumor suppressor, which p lypeptide is capable of producing a d tectable signal, and which DNA sequence is c upled to, and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the oncogene or the tumor suppressor gene, causes a measurable detectable signal to be produced by the polypeptide so expressed. The amount of the signal produced, is quantitatively determined and the amount so determined compared with the amount of produced signal detected in the absence of any molecule being tested or upon contacting the sample with any other molecule. So as to identify the molecule as one which causes a change in the detectable signal produced by the polypeptide so expressed, and thus identify the molecule as a molecule capable of transcriptionally modulating the expression of the oncogene or the tumor suppressor gene.

Additionally the invention provides a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of an oncogene or a tumor suppressor gene. The method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. The cells so contacted comprise DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the oncogene or the tumor suppressor gene, (ii) a promoter of the oncogene or the tumor suppressor gene, and (iii) a reporter gene, which expresses a polypeptide, coupled to, and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the oncogene or the tumor suppressor gene, causes a measurable change in the

amount of th p lypeptid produced. The am unt of th polypeptid so produced, is quantitatively determined and the amount so determined is compared with the amount of polypeptide produced in the absence of any molecule being tested or upon contacting the sample with any other molecule. So as to identify the molecule as one which causes a change in the amount of the polypeptide expressed, and thus identify the molecule as a molecule capable of transcriptionally modulating the expression of the oncogene or the tumor suppressor gene.

A method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of an oncogene or a tumor suppressor gene, is also provided. This method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. Each of the cells so contacted comprises DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the oncogene or the tumor suppressor gene, (ii) a promoter of the oncogene or the tumor suppressor gene, and (iii) a DNA sequence transcribable into mRNA coupled to and under the control of, the promoter. The contacting is effected under conditions such that the molecule, if capable of acting as a transcriptional modulator of the oncogene or the tumor suppressor gene, causes a measurable difference in the amount of mRNA transcribed from the DNA sequence. The amount of the mRNA produced is quantitatively determined and the amount so determined compared with the amount of mRNA detected in the absence of any molecule being tested or upon contacting the sample with any other molecule so as to identify the molecule as one which causes a change in the detectable mRNA amount of, and thus identify the molecul as a molecule capable of

transcriptionally modulating the expression of the ncog ne or tumor suppressor gene.

A screening method which comprises separately contacting each of a plurality of substantially identical samples, each sample containing a predefined number of cells under conditions such that contacting is affected with a predetermined amount of each different molecule to be tested, is also provided.

Further provided is a method of essentially simultaneously screening molecules to determine whether the molecules are capable of transcriptionally modulating one or more oncogenes or tumor suppressor genes according to the methods mentioned above.

Further provided is a method for directly transcriptionally modulating in a multicellular organism the expression of an oncogene or a tumor suppressor gene, the expression of which is associated with a defined physiological or pathological effect in the organism. This method comprises administering to the organism a molecule at a concentration effective to transcriptionally modulate expression of the gene and thus affect the defined physiological or pathological effect. In this method the molecule (a) does not naturally occur in the organism, (b) specifically transcriptionally modulates expression of the oncogene or the tumor suppressor gene, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

Brief Description of th Figures

Figure 1 is a view of the mammalian xpression shuttle vector pUV102 with its features. The mammalian expression shuttle vector was designed to allow the construction of the promoter-reporter gene fusions and the insertion of a neomycin resistance gene coupled to the herpes simplex virus thymidine kinase promoter (TK-NEO).

Figure 2 is a partial restriction enzyme cleavage map of the plasmid pD0432 which contains the luciferase gene from the firefly, Photinus pyralis.

Figure 3 is a partial restriction enzyme cleavage map of the plasmid pSVLuci which contains the luciferase gene from the firefly, Photinus pyralis.

Figure 4 is a partial restriction enzyme cleavage map of the plasmid pMLuci which contains the luciferase gene of the firefly, Photinus pyralis and the mouse mammary tumor virus long terminal repeat.

Figure 5 provides the nucleotide sequences of six oligonucleotides, pUV-1 through pUV-6, which were annealed, ligated, and inserted into the SalI/EcoR1 sites of the plasmid pTZ18R.

Figure 6 is a diagrammatic representation of the construction of the plasmid pUV001 from the plasmids pTZ18R and pBluescript KS(+).

Figure 7 is a diagrammatic representation of the construction of the plasmid pUV100 from the plasmid pUV001 and two DNA fragments, the XbaI/XmaI fragment from

pMLuci and the XmaI/BamHI fragment fr m pMSG.

Figure 8 is a diagrammatic repres ntation f the construction of the plasmid pUV100-3 from the plasmid pUV100 and a 476 b fragment containing a dimeric SV40 polyadenylation site.

Figure 9 is a diagrammatic representation of the construction of the plasmids pUV102 and pUV103 from the plasmid pUV100-3 and D-link oligonucleotides and the plasmid pUV100-3 and R-link oligonucleotides, respectively.

Figure 10 provides the nucleotide sequences of oligos 1-4 used for the construction of a synthetic HSV-thymidine kinase promoter and provides a diagrammatic representation of the HSV-TK promoter.

Figure 11 is a diagrammatic representation of the construction of the plasmid pTKL100 which contains the luciferase gene from the firefly, Photinus pyralis and the HSV-TK promoter sequence.

Figure 12 is a diagrammatic representation of the construction of the plasmid pTKNEO which contains the neo gene, from about 3.5 kb NheI/XmaI fragment from pTKL100, and the about 0.9 kb BstBI/BglII fragment containing the neo coding region from pRSVNEO.

Figure 13 is a diagrammatic representation of the construction of the plasmid pTKNEO2 from the plasmid pTKNEO and the oligonucleotides Neo 1 and 2.

Figure 14 is a diagrammatic representation of the construction of the plasmid pTKNEO3 from the plasmid

PTKNEO2 and about 0.9 kb EcoRl/SalI fragment from pMClNEO.

Figure 15 is a partial restriction enzyme cleavage map of the plasmid pNEU106 which contains neu upstream sequences fused to the luciferase coding region.

Figure 16 is a partial restriction enzyme cleavage map of the plasmid pKRAS106 which contains K-ras upstream sequences fused to the luciferase gene from the firefly, Photinus pyralis.

Figure 17 is a partial restriction enzyme cleavage map of the plasmid pHRAS106 which contains human H-ras upstream sequences fused to the luciferase gene from the firefly, Photinus pyralis.

Figure 18 is a partial restriction enzyme cleavage map of the plasmid pNRAS106 which contains human N-ras upstream sequences fused to the luciferase gene from the firefly, Photinus pyralis.

Figure 19 is a diagrammatic representation of the phl gene. Shown are EcoRI sites and genomic fragments used in defining the phl promoter.

Figure 20 is a diagrammatic representation of the various phl-luciferase fusion plasmids used to define the phl promoter. Plasmid 4 was used to create the stably transfected phl reporter cell line.

Figure 21 is a partial restriction enzyme cleavage map of the plasmid pP531106 which contains human p53 P1 promoter upstream sequences fused to the luciferase gene from the firefly, Photinus pyralis.

transcriptionally modulating the expression of the oncogen or tumor suppressor gene.

A screening method which comprises separately contacting each of a plurality of substantially identical samples, each sample containing a predefined number of cells under conditions such that contacting is affected with a predetermined amount of each different molecule to be tested, is also provided.

Further provided is a method of essentially simultaneously screening molecules to determine whether the molecules are capable of transcriptionally modulating one or more oncogenes or tumor suppressor genes according to the methods mentioned above.

Further provided is a method for directly transcriptionally modulating in a multicellular organism the expression of an oncogene or a tumor suppressor gene, the expression of which is associated with a defined physiological or pathological effect in the organism. This method comprises administering to the organism a molecule at a concentration effective to transcriptionally modulate expression of the gene and thus affect the defined physiological or pathological effect. In this method the molecule (a) does not naturally occur in the organism, (b) specifically transcriptionally modulates expression of the oncogene or the tumor suppressor gene, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

Brief Description of the Figures

Figure 1 is a view of the mammalian xpression shuttle vector pUV102 with its features. The mammalian expression shuttle vector was designed to allow the construction of the promoter-reporter gene fusions and the insertion of a neomycin resistance gene coupled to the herpes simplex virus thymidine kinase promoter (TK-NEO).

Figure 2 is a partial restriction enzyme cleavage map of the plasmid pD0432 which contains the luciferase gene from the firefly, Photinus pyralis.

Figure 3 is a partial restriction enzyme cleavage map of the plasmid pSVLuci which contains the luciferase gene from the firefly, Photinus pyralis.

Figure 4 is a partial restriction enzyme cleavage map of the plasmid pMLuci which contains the luciferase gene of the firefly, Photinus pyralis and the mouse mammary tumor virus long terminal repeat.

Figure 5 provides the nucleotide sequences of six oligonucleotides, pUV-1 through pUV-6, which were annealed, ligated, and inserted into the SalI/EcoR1 sites of the plasmid pTZ18R.

Figure 6 is a diagrammatic representation of the construction of the plasmid pUV001 from the plasmids pTZ18R and pBluescript KS(+).

Figure 7 is a diagrammatic representation of the construction of the plasmid pUV100 from the plasmid pUV001 and two DNA fragments, the XbaI/XmaI fragment from

pMLuci and th XmaI/BamHI fragm nt fr m pMSG.

Figure 8 is a diagrammatic repr sentation of the construction of the plasmid pUV100-3 from the plasmid pUV100 and a 476 b fragment containing a dimeric SV40 polyadenylation site.

Figure 9 is a diagrammatic representation of the construction of the plasmids pUV102 and pUV103 from the plasmid pUV100-3 and D-link oligonucleotides and the plasmid pUV100-3 and R-link oligonucleotides, respectively.

Figure 10 provides the nucleotide sequences of oligos 1-4 used for the construction of a synthetic HSV-thymidine kinase promoter and provides a diagrammatic representation of the HSV-TK promoter.

Figure 11 is a diagrammatic representation of the construction of the plasmid pTKL100 which contains the luciferase gene from the firefly, Photinus pyralis and the HSV-TK promoter sequence.

Figure 12 is a diagrammatic representation of the construction of the plasmid pTKNEO which contains the neo gene, from about 3.5 kb NheI/XmaI fragment from pTKL100, and the about 0.9 kb BstBI/BglII fragment containing the neo coding region from pRSVNEO.

Figure 13 is a diagrammatic representation of the construction of the plasmid pTKNEO2 from the plasmid pTKNEO and the oligonucleotides Neo 1 and 2.

Figure 14 is a diagrammatic representation of the construction of the plasmid pTKNEO3 from the plasmid

PTKNEO2 and about 0.9 kb EcoRl/SalI fragment fr m pMClNEO.

Figure 15 is a partial restriction enzyme cleavage map of the plasmid pNEU106 which contains neu upstream sequences fused to the luciferase coding region.

Figure 16 is a partial restriction enzyme cleavage map of the plasmid pKRAS106 which contains K-ras upstream sequences fused to the luciferase gene from the firefly, Photinus pyralis.

Figure 17 is a partial restriction enzyme cleavage map of the plasmid pHRAS106 which contains human H-ras upstream sequences fused to the luciferase gene from the firefly, Photinus pyralis.

Figure 18 is a partial restriction enzyme cleavage map of the plasmid pNRAS106 which contains human N-ras upstream sequences fused to the luciferase gene from the firefly, Photinus pyralis.

Figure 19 is a diagrammatic representation of the phl gene. Shown are EcoRI sites and genomic fragments used in defining the phl promoter.

Figure 20 is a diagrammatic representation of the various phl-luciferase fusion plasmids used to define the phl promoter. Plasmid 4 was used to create the stably transfected phl reporter cell line.

Figure 21 is a partial restriction enzyme cleavage map of the plasmid pP531106 which contains human p53 P1 promoter upstream sequences fused to the luciferase gene from the firefly, Photinus pyralis.

Figure 22 is a partial restricti n enzyme cleavage map of the plasmid pP532106 which contains human p53 P2 promoter upstream s quences fused to the lucif rase gen from the firefly, Photinus pyralis.

Figure 23 is a partial restriction enzyme cleavage map of the plasmid pCM106 which contains the Cytomegalovirus immediate early promoter fused to the luciferase gene from the firefly, Photinus pyralis.

Figure 24 is a partial restriction enzyme cleavage map of a human neu minigene which contains the neu upstream regulatory elements fused to the neu cDNA.

Figure 25 is a partial restriction enzyme cleavage map of a human K-ras minigene which contains the K-ras upstream regulatory elements fused to the neu cDNA.

Figure 26 is a graphical representation of the decay of reporter gene signal after treatment of cells with Actinomycin D. Plotted is relative intensity of the signal versus time after ActD addition.

Figure 27 is an autoradiogram of a Southern blot showing the correct luciferase vector integration of five independently isolated H-ras transfectants. Lanes 1 and 2 are plasmid controls. The expected result is a single band of the same molecular weight as the control.

Figure 28 is an autoradiogram of PCR reactions detecting varying amounts of M-CSF mRNA and a constant amount of lambda DNA.

Figure 29 is an interpretation of the data presented in Figur 28. Relativ band intensity of the M-CSF band is

plott d against th total RNA.

Figure 30 is an thidium bromide stain d gel showing the reaction products of PCR reactions designed to specifically detect N-ras, neu, K-ras and H-ras mRNAs. 10 and 0.4 ng of U5637 total RNA was used. In each sample a sample of phage lambda DNA and lambda control oligos was added to control for the efficiency of the PCR reaction. This is the uppermost, consistent band in each lane. In each case the oligos specifically amplifies a DNA fragment of the correct molecular weight. A few extra bands of unknown origin appear in the c-erbB2 lanes and in the lane representing the highest concentration of H-ras mRNA.

Figure 31 is a quality assurance analysis of a high throughput screen measuring the ratios of negative values at various positions within a plate. The expected value is 1.0.

Figure 32 is a quality assurance analysis of a high throughput screen measuring a coefficient of variance for the negative controls on a number of plates. Values less than 10 are acceptable.

Figure 33 is a quality assurance analysis of a high throughput screen measuring a coefficient of variance for the positive controls on a number of plates. Values less than 10 are acceptable.

Figure 34 is a quality assurance analysis of a high throughput screen measuring a response of a reporter cell line to three different concentrations of a compound known to induce transcription.

Detailed Description of the Invention

As used in this application, the following words or phrases have the meanings specified.

Antisense nucleic acid means an RNA or DNA molecule or a chemically modified RNA or DNA molecule which is complementary to a sequence present within an RNA transcript of a gene.

Directly transcriptionally modulate the expression of a gene means to transcriptionally modulate the expression of the gene through the binding of a molecule to (1) the gene (2) an RNA transcript of the gene, or (3) a protein which binds to (i) such gene or RNA transcript, or (ii) a protein which binds to such gene or RNA transcript.

A gene means a nucleic acid molecule, the sequence of which includes all the information required for the normal regulated production of a particular protein, including the structural coding sequence, promoters and enhancers.

Indirectly transcriptionally modulate the expression of a gene means to transcriptionally modulate the expression of such gene through the action of a molecule which cause enzymatic modification of a protein which binds to (1) the gene or (2) an RNA transcript of the gene, or (3) protein which binds to (i) the gene or (ii) an RNA transcript of the gene. For example, altering the activity of a kinase which subsequently phosphorylates and alters the activity of a transcription factor constitutes indirect transcript modulation.

Ligand means a molecule with a molecular weight of less

than 5,000, which binds to a transcription factor f r a gene. Th binding of the ligand to the transcription factor transcriptionally modulates the expression of the gene.

Ligand binding domain of a transcription factor means the site on the transcription factor at which the ligand binds.

Modulatable transcriptional regulatory sequence of a gene means a nucleic acid sequence within the gene to which a transcription factor binds so as to transcriptionally modulate the expression of the gene.

Oncogene means a normal cellular gene which has been activated by one of several possible mechanisms, including point mutations, translation, amplification or overexpression contributes to the malignant phenotype of a cancer cell.

Receptor means a transcription factor containing a ligand binding domain.

Specifically transcriptionally modulate the expression of a gene means to transcriptionally modulate the expression of such gene alone, or together with a limited number of other genes.

Transcription means a cellular process involving the interaction of an RNA polymerase with a gene which directs the expression as RNA of the structural information present in the coding sequences of the gene. The process includes, but is not limited to the following steps: (1) the transcription initiation, (2) transcript elongation, (3) transcript splicing, (4) transcript

capping, (5) transcript termination, (6) transcript polyadenylation, (7) nuclear export of the transcript, (8) transcript editing, and (9) stabilizing the transcript.

Transcription factor for a gene means a cytoplasmic or nuclear protein which binds to (1) such gene, (2) an RNA transcript of such gene, or (3) a protein which binds to (i) such gene or such RNA transcript or (ii) a protein which binds to such gene or such RNA transcript, so as to thereby transcriptionally modulate expression of the gene.

Transcriptionally modulate the expression of a gene means to change the rate of transcription of such gene.

Triple helix means a helical structure resulting from the binding of one or more oligonucleotide to double stranded DNA.

Tumor suppressor gene means a normal cellular gene, the loss of whose function contributes to the malignant phenotype of a cancer cell.

The invention provides a method of directly transcriptionally modulating the expression of an oncogene or a tumor suppressor gene, the expression of which is associated with a defined physiological or pathological effect within a multicellular organism. This method comprises contacting a cell, which is capable of expressing the gene, with a molecule at a concentration effective to transcriptionally modulate expression of the gene and thereby affect the level of the oncogene or the tumor suppressor gene product encoded by the gene which is expressed by the cell. In this

method the molecule (a) does not naturally occur in the cell, (b) specifically transcriptionally modulates expression of the oncogene or the tumor suppressor gene, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

In one embodiment the molecule does not naturally occur in any cell of a lower eucaryotic organism such as yeast.

In a preferred embodiment, the molecule does not naturally occur in any cell, whether of a multicellular or a unicellular organism. Alternatively, the molecule is naturally occurring, but not normally found in the cell. In a presently more preferred embodiment, the molecule is not a naturally occurring molecule, e.g. is a chemically synthesized entity.

The cell in the above methods may be a cell of the multicellular organism, such as an animal cell, e.g. a human cell.

The method of the invention permits modulation of the transcription of the gene which results in upregulation or downregulation of expression of the oncogene or the tumor suppressor gene, depending on the identity of the molecule which contacts the cell.

In one embodiment the molecule binds to a modulatable transcription sequence of the gene. For example, the molecule may bind to a promoter region upstream of the coding sequence encoding the oncogene or the tumor

suppr ssor gene.

In one mbodim nt of the method of th inv ntion the molecule comprises an antisense nucleic acid which is complementary to a sequence present in a modulatable, transcriptional sequence. The molecule may also be a double-stranded nucleic acid or a nucleic acid capable of forming a triple helix with a double-stranded DNA.

In accordance with the method of this invention, the oncogene may be a mutant c-neu gene, a mutant c-K-ras gene, a mutant c-H-ras gene, a mutant c-N-ras gene, a phl-abl gene fusion, a mutant myc gene, a mutant fms gene, a mutant erbA gene, a retroviral oncogene or a mutant p53 gene. The tumor suppressor gene may be a p53 gene, a retinoblastoma gene, a Wilms' tumor gene, a neurofibromatosis type 1 gene, a DCC gene, an erbA gene, or an adenomatous polyposis coli gene.

The invention further provides a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of an oncogene or the tumor suppressor gene. This method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the oncogene or the tumor suppressor gene, (ii) a promoter of the oncogene or the tumor suppressor gene, and (iii) a DNA sequence encoding a polypeptide other than the oncogene or the tumor suppressor gene product, which polypeptide is capable of producing a detectable signal and which DNA sequence is coupled to, and under the control of, the promoter, under conditions such that the

molecule, if capable of acting as a transcriptional modulator of th oncogene or the tumor suppressor g n , causes a measurabl detectable signal to b pr duced by the polypeptide so expressed. The amount of the signal produced is quantitatively determined and the amount so determined compared with the amount of produced signal detected in the absence of any molecule being tested or upon contacting the sample with any other molecule so as to identify the molecule as one which causes a change in the detectable signal produced by the polypeptide so expressed, and identify the molecule as a molecule capable of transcriptionally modulating the expression of the oncogene or the tumor suppressor gene.

Additionally, the invention provides a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of an oncogene or a tumor suppressor gene. The method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. The cells comprise DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the oncogene or the tumor suppressor gene, (ii) a promoter of the oncogene or the tumor suppressor gene, and (iii) a reporter gene, which expresses a polypeptide, coupled to, and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the oncogene or tumor suppressor gene, causes a measurable change in the amount of the polypeptide produced. The amount of the polypeptide is quantitatively determined and compared with the amount of polypeptid pr duced in the abs nce of any molecule being tested or upon contacting the sample

with any other molecule so as to identify the molecule as one which causes a change in the amount of the polypeptide expressed, and thus identify the molecule as a molecule capable of transcriptionally modulating the expression of the oncogene or the tumor suppressor gene.

A method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of an oncogene or a tumor suppressor gene, is also provided. This method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. Each of the cells comprise DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the oncogene or the tumor suppressor gene, (ii) a promoter of the oncogene or tumor suppressor gene, and (iii) a DNA sequence transcribable into mRNA coupled to and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the oncogene or the tumor suppressor gene, causes a measurable difference in the amount of mRNA transcribed from the DNA sequence. The amount of the mRNA produced is quantitatively determined the amount so determined compared with the amount of mRNA detected in the absence of any molecule being tested or upon contacting the sample with any other molecule so as to identify the molecule as one which causes a change in the detectable mRNA amount of, and thus identify the molecule as a molecule capable of transcriptionally modulating the expression of the oncogene or the tumor suppressor gene.

In the above methods the sample preferably comprises cells in monolayers or suspension. The cells are typically animal cells, e.g. human cells. The predefined

number of cells is preferably from about 1 to about 5 X 10^5 cells, or from about 2 X 10^2 to about 5 X 10^4 cells. The predetermined am unt or concentration of the mol cule to be tested is typically based upon the volume of the sample, or be from about 1.0 pM to about 20 μM, or from about 10μM to about 500μM. The contacting may be effected from about 1 to about 24 hours, or from about 2 to about 12 hours, it may also be effected with more than one predetermined amount of the molecule to be tested.

The molecule to be tested may be a purified molecule. Additionally the modulatable transcriptional regulatory sequence may comprises a cloned genomic regulatory sequence. The DNA referred to in the above methods may consist essentially of more than one modulatable transcriptional regulatory sequence. The DNA sequence encoding the polypeptide may be inserted downstream of the promoter of the oncogene or tumor suppressor gene by homologous recombination.

In these methods the polypeptide may be a luciferase, chloramphenicol acetyltransferase, β glucuronidase, β galactosidase, neomycin phosphotransferase, alkaline phosphatase or guanine xanthine phosphoribosyltransferase. The polypeptide may be capable of complexing with an antibody, or biotin.

The mRNA may be detected by quantitative polymerase chain reaction.

A screening method which comprises separately contacting each of a plurality of substantially identical samples, each sample containing a predefined number of cells under conditions such that contacting is affected with a pred termined amount of each different molecule to be

t sted, is also included.

Th plurality of samples may c mprises more that about 10^4 samples, or more than about 5 X 10^4 samples.

Pursuant to the provisions of the Budapest Treaty on the International Recognition of Deposit of Microorganisms for Purpose of Patent Procedure, the plasmid and the cell lines listed below have been deposited with the American Type Culture Collection ("ATCC"), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A.,:

1. a plasmid designated pUV106, deposited under ATCC Accession No. 40946.

2. a human colon adenocarcinoma cell line, transfected with pHRA521, designated H21, deposited under ATCC Accession No. CRL 10640.

3. a HTB-30 human colon adenocarcinoma cell line, transfected with pNEU106, designated N-2, deposited under ATCC Accession No. CRL 10658.

4. a SW 480 human breast carcinoma cell line, transfected with pKRAS106, designated K-2, deposited under ATCC Accession No. CRL 10662.

5. a K562 cell line, transfected with pNEU106, designated bK108, deposited under ATCC Accession No. CRL 10665.

6. a NIH Swiss mouse embryo cell line, NIH 3T3, transfected with the MMTV reporter plasmid, designated M10, deposited under ATCC Accession No. CRL 10659.

Further provided is a method of essentially simultaneously screening molecules to determine whether the molecules are capable of transcriptionally modulating one or more oncogenes or tumor suppressor genes according to the methods described above.

A screening method where more than about 10^3 samples per week are contacted with different molecules is encompassed by this invention.

Further provided is a method for directly transcriptionally modulating in a multicellular organism the expression of an oncogene or a tumor suppressor gene, the expression of which is associated with a defined physiological or pathological effect in the organism. This method comprises administering to the organism an a molecule at a concentration effective to transcriptionally modulate expression of the gene and thus affect the defined physiological or pathological effect. In this method the molecule (a) does not naturally occur in the organism, (b) specifically transcriptionally modulates expression of the oncogene or the tumor suppressor gene, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

The molecule may bind to a modulatable transcription sequence of the gene. The molecule may comprise an antisense nucleic acid, a double-stranded nucleic acid molecule, a nucleic acid capable of forming a triple helix with double-stranded DNA. The multicellular

organism may be an animal including a human being.

Th physiological effect may b the protection of non-neoplastic cells from damage by chemotherapeutic agents and the defined pathological effect may be a disorder and modulated expression of an oncogene or tumor suppressor gene is associated with amelioration of the disorder. The defined pathological effect may be cancer or leukemia. The cancer may include malignant melanoma, lung cancer, colon cancer, pancreatic cancer, ovarian cancer or breast cancer.

The administering in the preceeding method may comprise topical contact, oral, transdermal, intravenous, intramuscular or subcutaneous administration.

Methods of administration of molecules in the practice of the invention are well known to those skilled in the art as are methods of formulating the molecule for administration depending on the specific route of administration being employed.

This invention is illustrated in the Experimental Detail section which follow. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

EXPERIMENTAL DETAILS

MATERIALS AND METHODS

A. Cell Culture

All media and reagents used for routine cell culture were purchased from Gibco (Grand Island, NY), Hazelton (Lenexa, KS), or Whittaker M.A. Biologicals (Walkersville, MD). Fetal calf serum (FCS) was from Hyclone (Logan, UT).

A human colon adenocarcinoma cell line, SW480 (ATCC CCL 228) was used for experiments concerning expression of the three ras proto-oncogenes and the p53 tumor suppressor gene. This cell line was maintained on DMEM, 15% fetal calf serum (FCS), 1% Nonessential amino acids (NEAA). Stable transfectants of this cell line were selected in the same medium with the addition of G418 (Geneticin, Gibco) to a final concentration of 0.6 mg/ml.

A human breast adenocarcinoma derived cell line, SK-BR-3 (ATCC HTB 30) was used for the experiments concerning expression of the neu (erbB2) proto-oncogene. This cell line was maintained on DMEM, 15% FCS and 1 μg/ml insulin. Stable transfectants of this cell line were selected in this same medium with the addition of G418 to a final concentration of 0.4 mg/ml.

A human chronic myelogenous leukemia derived cell line, K562 (ATCC CCL 243) was used for experiments concerning the expression of the phl promoter.

A human hepatocellular carcinoma derived cell line, Hep3B (ATCC# HB8064), was used for transfection of plasmids

containing the cytomegalovirus (CMV) promoter (used as a control for th high throughput screen). These cells wer maintained on MEM:OptiMEM (1:1) supplemented with 10% FCS.

A murine embryonic fibroblast cell line, NIH3T3 (ATCC# CCL92), was used for the transfection of plasmids carrying the MMTV promoter (a control cell line). These cells were maintained on DMEM, supplemented with 10% FCS.

B. Construction of the Luciferase-Fusion Reporter Vector

Unless otherwise indicated, molecular cloning procedures were performed essentially according to Maniatis et al. (42). Oligonucleotides were synthesized by the beta-cyanoethyl phosphoramidite method according to protocols provided by the manufacturer of the DNA-synthesizer (Model 380A, Applied Biosystems (Foster City, CA).

A mammalian expression shuttle vector was designed to allow the construction of the promoter-reporter gene fusions to be used in high-throughput screens to identify transcriptionally modulating chemicals. Features of the plasmid are shown in Figure 1. The shuttle vector was constructed in several steps.

The firefly luciferase gene was removed from the plant expression plasmid pDO432 (111) (Figure 2) as a 1.9 kb BamHI fragment and cloned into the BamHI site of pSVL (Pharmacia, Piscataway, NJ), a mammalian expression vector containing the SV40 promoter. The resulting plasmid (pSVLuci; Figure 3) was digested with XhoI and SalI to produce a 2.4 kb fragment containing the luciferase coding sequ nces and the SV40 late

p lyadenylation site. This fragment was inserted into the XhoI site of pMSG (Pharmacia, Piscataway, NJ), a eucaryotic expression vector containing the MMTV promoter. The resulting MMTV promoter-luciferase fusion plasmid (pMLuci; Figure 4) was used to transfect NIH/3T3 cells as described below. Similar constructs can be made using luciferase vectors from Clontech (Palo Alto, CA).

Six oligonucleotides (pUV-1 through pUV-6) were synthesized (see Figure 5 for sequence)(SEQ ID NO: 1-6). The sequences of pUV-1, pUV-2 and pUV-3 correspond to a multicloning site, the beta-globin leader sequence and the first 53 bases of the firefly luciferase coding region. The sequences of pUV-4, pUV-5 and pUV-6 are complementary to the first three oligonucleotides. The pUV oligonucleotides were annealed, ligated and inserted into the SalI/EcoRI sites of pTZ18R (Pharmacia, Piscataway NJ) (Figure 6). The resulting vector was then digested with SmaI/PvuII and the oligonucleotide containing fragment was cloned into the pBluescriptKS(+) plasmid (Stratagene, La Jolla, CA), previously digested with PvuII, to yield pUV001 (Figure 6). Several fragments were ligated into pUV001 to create pUV100. The luciferase coding sequences (except first 53 bases) and polyadenylation site were obtained as a 1.8 kilobase XbaI/XmaI fragment from pMLuci (section B-1, Figure 4). The SV40 early splice site and the SV40 late polyadenylation site were obtained as an 871 bp XmaI/BamHI fragment from pMSG (Pharmacia, Piscataway NJ, Figure 7). Both DNA fragments were cloned into pUV001, previously digested with XbaI/BamHI to yield pUV100 (Figure 7).

A 476 bp fragment containing a dimeric SV40 polyadenylation site was then cl ned into the BclI site

of pUV100 (Figure 8). To do this, a 238 bp BclI/BamHI fragment was obtain d from SV40 genomic DNA (BRL), ligat d, digest d with BclI/BamHI, gel isolated, and inserted into pUV100, resulting in the vector pUV100-3 (Figure 8). Linkers containing one SfiI and one NotI restriction site were then cloned into the PvuII/BamHI sites of pUV100-3. Two sets of linkers were synthesized containing the SfiI site in opposite orientations (oligonucleotides D-link1 and D-link2 and oligonucleotides R-link1 and R-link2). The sequences of the oligonucleotides (SEQ ID NO: 7-10) were:

5' GATCGGCCCCTAGGGCCGCGGCCGCAT 3' (D-link1)
5' ATGCGGCCGCGGCCCTAGGGGCC 3' (D-link2)
5' GATCGGCCCTAGGGGCGGCCGCAT 3' (R-link1)
5' ATGCGGCCGCGGCCCCCTAGGGCC 3' (R-link2)

The plasmid that contains D-link oligonucleotides was named pUV102 and the plasmid that contains R-link oligonucleotides was named pUV103 (Figure 9).

The neomycin resistance gene (neo) was then placed under control of the Herpes Simplex Virus thymidine kinase (HSV-TK) promoter to generate a resistance cassette which is free of known enhancer sequences. To do this the HSV-TK promoter was synthesized using four oligonucleotides (Figure 10) (SEQ ID NO: 11-14) designed according to published sequence information (44), and including an SfiI restriction site 5' of the HSV-TK sequences. These oligonucleotides were phosphorylated, annealed, ligated and inserted into pUV100 digested previously with HindIII/NheI, generating the vector pTKL 100 (Figure 11). After verifying the HSV-TK sequence, the about 3.5 kb NheI/SmaI fragment was isolated from pTKL100, and th about 0.9 kb BstBI/BglII fragment

containing the neo coding region was isolated from pRSVNEO (45). Th s two fragments were fill d in with Klenow polymerase and ligated to form pTKNEO (Figure 12). An additional SfiI site was then inserted 3' of the neo gene by isolating the about 1.8 kb SfiI/BamHI and about 2.6 kb SfiI/PVUII fragments of pTKNEO and conducting a three way ligation along with a synthesized SfiI oligonucleotide generating pTKNEO2 (Figure 13) (SEQ ID NO: 15-16). The HSV-TK/NEO vector containing an optimized Kozac sequence was also utilized (Stratagene, La Jolla, CA, pMC1NEO). An additional vector was constructed by replacing the about 0.9 kb EcoRI/SalI fragment of pTKNEO2 with the about 0.9 kb EcoRl/SalI fragment from pMC1NEO. This vector was termed pTKNEO3. (Figure 14). The SfiI fragment of pTKNEO3, containing the TK promoter and the neomycin resistance gene, was cloned into the SfiI site of pUV102 to yield pUV106.

C. Molecular Cloning of Oncogene and Tumor Suppressor Promoters and Insertion into the Mammalian Expression Shuttle Vector

Strategy: This section describes (a) the molecular cloning of the promoter and transcriptionally modulatable regulatory sequences of the human (I) neu (c-erbB2), (II) K-ras, (III) H-ras, (IV) N-ras proto-oncogenes, the (V) phl promoter (responsible for the expression of the phl-abl oncogene fusion following the translocation event characteristically observed in chronic myelogenous leukemia (generating the Philadelphia chromosome), and the (VI) p53 tumor suppressor gene, and (b) the making of constructs where these regulatory sequences control the expression of the firefly luciferase gene. These constructs were transfected into cells as described in below and analyzed for their utility as r porters for th

disc very of gene expression modulating comp unds (for xample, in a high-throughput screen to identify chemicals acting as sp cific transcriptional modulators.

To make such constructs, several kilobases of sequence upstream of the transcription start site, along with 5' untranslated sequences up to the translation start site (ATG), of a gene of interest were inserted 5' of the luciferase coding region, along with any additional sequences (e.g. intronic enhancers) required for properly regulated expression of the luciferase reporter. In this way constructs can be made where all sequences upstream of their translation start site are from the gene of interest, and all coding sequences are from the luciferase gene. How this was accomplished for the individual oncogenes is described below.

1. neu (c-erbB2) reporter vector

Oligonucleotide probes based on the published sequence (46) of the 5' region of the c-erbB2 gene were synthesized and used to screen a human leukocyte genomic library (Clontech Inc.). A 3.2 kb BglI fragment from a positive plaque, containing the upstream regulatory elements, the 5' untranslated leader and exon 1 was then subcloned into pBluscriptKS(+), generating pNEU001. A 1.8 kb HincII-NcoI fragment from pNEU001, containing the upstream regulatory elements and most of the 5' untranslated leader was purified by preparative gel electrophoresis and ligated into pUV103 previously digested with SnaBI and NcoI, generating pNEU002. Two oligonucleotides (SEQ ID NO: 17-18) were synthesized:

5'-CATGGGGCCGGAGCCGCAGTGAGCAC-3' and

5'-CATGGTGCTCACTGCGGCTCCGGCCC-3'

These oligonucleotides were annealed to one another, phosphorylated and ligated into NcoI digested pNEU002, generating pNEU103. The synthetic linker fuses the DNA coding for the neu 5' untranslated leader to the luciferase open reading frame such that the AUG utilized for translation initiation of the neu gene forms the first codon of the luciferase gene. The ScaI-XbaI fragment of pNEU103, containing vector sequences, the upstream regulatory elements, the 5'untranslated leader and a portion of the luciferase open reading frame, was purified by preparative gel electrophoresis and ligated into pUV106 which had previously been digested with ScaI and XbaI, generating pNEU106 (Figure 15). Linearized pNEU106 was used in the transfections to generate the neu-luciferase reporter cell lines as described below.

2. K-ras reporter vector

Oligonucleotides based on the published K-ras sequence (47) were used to isolate two genomic clones by standard methods from a human leukocyte library (Clontech). DNA from these two phages was subcloned into pBluscriptKS(+) (Stratagene) generating pKS4 and pKS11.

A 4 kb XhoI-StuI fragment of pKS11, containing most of intron 1 and exon 1 up to a point 11 bases 5' of the point of translation initiation, was isolated by preparative gel electrophoresis and ligated into XhoI-StuI digested pGEM7Zf (Promega) which had been previously modified by inserting an adaptor the ApaI and XhoI sites in the original vector. This adaptor comprised of two oligonucleotides (5'-TCGAGATCTGAGGCCTGCTGACCATGGGGGCC-3' and 5'-CCCATGGTCAGCAGGCCTCAGATC-3') (SEQ ID NO: 19-20) annealed to one another and was used to allow the proper alignment of the K-ras ATG initiator codon with the

luciferase ORF in the final construct (below). Th resulting plasmid was designat d pGEM715.

A 3 kb HinDIII-XhoI fragment from pKS4, comprising 2.2 kb of K-ras untranscribed upstream DNA and sequences coding for exon 0 and part of intron 1 was purified by preparative gel electrophoresis and ligated into pGEM715 which ad been previously digested with HinDIII and XhoI to generate pGEM7.

A 7.7 kb HinDIII-NcoI fragment of pGEM7, comprising 2.2 kb of K-ras upstream regulatory elements, exon 0, intron 1, and part of exon 1 (to the ATG at the NcoI site), was purified by preparative gel electrophoresis and ligated int pUV102 which had previously been digested with HinDIII and NcoI to generate pKRAS102. The TK-Neo fragment from pTKNeo3 was then ligated into the SfiI site of pKRAS102 to generate pKRAS106 (Figure 16), the vector used foe transfections to generated the stable reporter cell lines.

3. H-ras reporter vector

A 6.4 kb BamHI fragment, containing the entire H-ras gene was isolated from a genomic DNA library by conventional means. This BamHI fragment was subcloned into the BamHI site of pSV2Neo (48) generating pHRAS001. A 1.7 kb BamHI-MstII fragment containing the H-ras untranscribed regulatory elements and the 5' untranslated leader including intron-1 (up to within 9 bp of the sequences encoding the H-ras ATG translation initiation site) was purified by preparative gel electrophoresis, and along with an adaptor (5'-TGAGGAGC-3' and 5'-CATGGCTCC-3' annealed together) ligated into BglII-NcoI cut pUV102 g nerating pHRAS102. Th TK-Neo3 fragment of pTKNeo3 was

then ligated into th SfiI site of pHRAS102 to generate pHRAS106 (Figure 17), th vector used to generate the stable H-ras reporter cell line.

4. N-ras reporter vector

Oligonucleotides, based on the published sequence of the human N-ras gene (57) are synthesized and used to screen a human leukocyte genomic library (Clontech). Positive plaques are subcloned as 8 kb EcoRI fragments containing the upstream regulatory elements, 5' untranslated leader, exons 1 and 2, intron 1 and a portion of intron 2, into pBluscriptKS(+), generating pNRAS001. A 4.5 Kb NcoI-HinDIII fragment containing the upstream regulatory elements and most of the 5' untranslated leader, is purified by preparative gel electrophoresis and ligated along with a 75 bp synthetic HinDIII-NcoI linker, into pUV102 which had previously been digested with NcoI, generating pNRAS102. The SfiI pTKNeo3 fragment containing the neomycin resistance gene was then ligated into the SfiI site of pNRAS102, generating, pNRAS106 the final reporter vector (Figure 18).

5. phl-abl reporter vector

For the isolation of the phl promoter, five oligonucleotides were synthesized which were complementary to regions within the first exon. These oligonucleotides were used to screen a human leukocyte genomic library (Clontech). Positive clones were restriction mapped, subcloned and sequenced. One subclone contained 534 bases upstream of the putative transcription start site (based on a published cDNA sequence), the region corresponding to the long 5' untranslat d leader (472 bases), and 175 bases of the

coding region in the first exon (Figure 19). This subclone was used to construct a series of four promoter-luciferase reporter vectors, each with a different amount of 5' upstream DNA. These plasmids are illustrated in Figure 20. Plasmid 1 consists of the 4 kb Xba I fragment ligated into Xba I-Spe I digested pUV103. Plasmid 2 consists of the 1.2 kb Bgl II-Xba I fragment ligated into Bgl II-Xba I digested pUV103. Plasmid 3 consists of Bal I-SnaB I deletion of Plasmid 2. Plasmid 4 consists of the 135 bp Xma I-Xba I fragment inserted into pUV103.

6. p53 reporter vector

The arrangement of promoters, 5' untranslated exons and a large (10 kb) first intron precludes the construction of a p53 reporter vector which includes all of the promoter elements and the complete untranslated leader such that the AUG initiator codon of the normal p53 ORF becomes the first codon of the luciferase ORF. As an alternative, fusion constructs which are similar to those successfully employed by others for the analysis of the expression of this gene are used. There are two promoters which play a role in the regulated expression of the p53 tumor suppressor gene. Because it is not yet clear which of these two promoters is the most important for determination of the cellular level of the p53 protein, two plasmids are constructed, each fusing one of the promoters to the luciferase open reading frame. Oligonucleotides based on the published sequence are used as probes to isolate genomic p53 clones from a human leukocyte library (Clontech). Subclones of this original clone serve as a source of the fragments used to construct both of the p53 reporter vectors. The p53P1 containing region is isolated as a 2.4 kb EcoRI-XbaI fragment essentially as described in reference (49). This

fragment is rendered blunt ended by Klenow fragment and ligated into SnaBI digested pUV106, the proper orientation of the fragment is confirmed by restriction mapping, generating pP531106 (Figure 21). The p532 promoter is isolated as a 1.4 kb XbaI-BglII fragment, essentially as described in (50). The XbaI site is rendered blunt ended with Klenow fragment prior to the secondary digestion by BglII. The resulting fragment is purified by preparative gel electrophoresis, and this fragment is then ligated into pUV106 which had previously been digested with SnaBI and BglII, generating pP532106 (Figure 22).

7. CMV reporter vector

A 580 bp cytomegalovirus genomic fragment containing the immediate early promoters and enhancers (51) was ligated into pUV100 previously digested with NotI and NheI and rendered blunt ended by treatment with Klenow fragment, generating pUVCM. An 888 bp NaeI-XbaI fragment from pUVCM, including the CMV promoter and enhancers plus a portion of the luciferase coding region, was purified by preparative gel electrophoresis and ligated into pUV106 which had previously been digested with SnaBI and XbaI, generating pCM106 (Figure 23), the vector used to transfect the CMV reporter cell lines.

D. Construction of Mini-genes

As a secondary analysis of compounds identified as able to repress the synthesis of specific oncogenes, a series of "mini-genes" were constructed. A mini-gene is the fusion of promoter elements identical to those used to construct luciferase reporter vectors, fused to the cDNA of the activated oncogene. Thes constructs, when

transfect d into an appropriate host cell line, lead to its ph notypic transformation. Comp unds which repress the activity of th r gulatory elem nts lead to a reversal of the transformed phenotype. The construction of the c-erbB2 and K-ras minigenes are described below.

1. c-erbB2 minigene

The 1.7 kb erbB2 promoter fragment (SalI-NcoI) was purified from pNEU102 and combined an NcoI-SalI cDNA fragment isolated form a commercially available breast carcinoma cDNA library (Clontech) according to the supplier's directions. A 26 bp linker was inserted into the NcoI site to correctly fuse the untranslated leader to the ATG initiation codon (Figure 24).

2. K-ras minigene

The K-ras promoter fragment was isolated as a HinDIII-StuI fragment from pKRAS106. A K-ras cDNA containing plasmid was obtained from the ATCC (ATCC# 41027) and mutated by oligonucleotide mutagenesis (Ammersham) to generate a cDNA encoding a mutant K-ras protein with an aspartic acid at position 12. A StuI-XhoI fragment containing the K-ras coding region was ligated to the promoter fragment, generating the final minigene (Figure 25).

E. Liquid Scintillation Counter Bioluminescence Assay

To assay for luciferase expression in transient expression assays in the various transfected clones (see below), cells were incubated with various transcriptional inducers in serum free defined media, washed 3 times with Dulbecco's phosphat -buff r d saline (D-PBS, Gibc) and

lys d in Lysis Buffer 1 (50 mM Tris acetate pH 7.9, 1 mM EDTA, 10 mM magn sium acetate, 1 mg/ml b vine serum albumin [BSA], 0.5% Brij 58, 2 mM ATP, 100 mM dithiothreitol [DTT]). All reagents were obtained from Sigma except for DTT which was from Boehringer Mannheim. After lysis, cell debris was sedimented by brief centrifugation, and 950 µl of supernatant extract were added to a glass scintillation vial. Samples were counted individually in an LKB (Gaithersburg, MD) scintillation counter on a setting which allows measurement of individual photons by switching off the coincidence circuit. The reaction was started by addition of 50 µl of 2mM luciferin (Sigma, St. Louis, MO or Boehringer Mannheim, Indianapolis IN) in Buffer B (Buffer B-Lysis Buffer 1 without Brij 58, ATP and DTT) to the 950 µl of lysate. Measurement was started 20 seconds after luciferin addition and continued for 1 minute. Results were normalized to protein concentration using the Bradford protein assay (BioRad, Richmond CA) or to cell numbers using Trypan Blue (Sigma) exclusion counting in a hemocytometer.

F. Transfection

Cell were transfected by one of three methods, following manufacturer's instructions; by calcium phosphate precipitation (Pharmacia), lipofection (Life Technologies Inc.) or electroporation (BioRad). In most cases, 25-75 µg of plasmid DNA, linearized by a single restriction endonuclease cut within the vector sequences, was electroporated into approximately 5 million cells. When co-transfection of a separate neomycin resistant plasmid was employed the molar ratio of luciferase fusion plasmid to neomycin resistant plasmid was either 10:1 or 20:1. Neomycin resistant clones were s lected by growth in

m dia containing G418 (Gen ticin, Gibco).

G. Southern blotting

To monitor correct and complete stable integration of transfected promoter/reporter constructs, stably transfected cell clones were subjected to Southern blot analysis (52). Genomic DNA was prepared of each clone to be tested and restriction-cut with DraI or another appropriate restriction endonuclease. After electrophoresis, transfer to nylon filters and immobilization by UV irradiation using a Stratalinker UV device (Stratagene, La Jolla, California), integrated promoter/luciferase fusion constructs were visualized by probing with radioactively labelled XbaI-EcoRI fragments of the luciferase coding region. Probes were labelled using the random primer method (53). Since DraI cuts in the SV40 polyadenylation sites located in the mammalian expression shuttle vector just upstream the inserted promoter sequences as well as downstream of the luciferase coding region, but not in most of the promoter sequences used for generating stably transfected cell clones, a single fragment should be visualized by the probe used. The size of that fragment should be characteristic for each of the promoter sequences analyzed.

H. Isolation of Total Cellular RNA

Total cellular RNA was isolated from the luciferase-fusion containing cell clones or from untransfected host cells following incubation with various transcriptionally modulating chemicals known previously to affect gene expression. Total cellular RNA was isolated using the RNAZol method (CINNA/BIOTECX, Friendswood, TX,

Lab ratories International, Inc.). Cells were resuspended and lysed with RNAZol s lution (1.5 ml/9 cm petri dish) and the RNA was solubilized by passing the lysate a few times through a pipette. Chloroform was added to the homogenate (0.1 ml/1.0 ml), and samples were shaken for 15 seconds followed by a 5 minute incubation on ice. After centrifuging for 10 minutes, the upper phase was collected and an equal volume of isopropanol was added. Samples were incubated for 45 minutes at -20°C, and the RNA was pelleted for 15 minutes at 12,000 x g at 4°C. The RNA pellet was then washed with 70% ethanol and dried briefly under vacuum.

I. Polymerase Chain Reaction

Total RNA was isolated using the approach described above, first strand cDNA generated by either oligo dT, random hexamer or gene specific oligonucleotide priming. Specific amplification oligonucleotides were added, and the polymerase chain reaction carried out according to established methods (54).

RNA levels are quantitated by established methods (55) which include the addition of varying amounts of a control RNA and thereby establishing a standard curve. PCR products are visualized on an ethidium bromide stained agarose gel and are quantitated by measuring the incorporation of radiolabelled deoxynucleotide triphosphates using liquid scintillation.

J. Specific PCR mRNA Detection and Quantitation of Oncogenes and Tumor Suppressor Genes

Oligonucle tides (SEQ ID NO: 21-34) were designed for th

specific detection of each of the genes to be analyzed:

K-ras:	5'-CTCTTGGATATTCTCGACACACAGCA-3'
	5'-GTGTCTACTGTTCTAGAAGG-3'
H-ras:	5'GCACGCACTGTGGAATCTCGG-3'
	5'-ACGTCGAGTACGTCGGCCCCGGTG-3'
N-ras:	5'-GCCCACGAACTGGCCAAGAGTT-3'
	5'-CTGAGTCCCATCATCACTGCTCTT-3'
neu:	5'-CCAATGCCAGCCTGTCCTTCCTGCAG-3'
	5'-GATCAAGACCCCTCCTTTCAAGATCT-3'
phl-abl:	5'-ATTCCGCTGACCATCAATAAGGAAG-3'
	5'-ATAGCCTAAGACCCGGAGCTTTTCAC-3'
p53:	5'-GATGGAGAATATTTCACCCTTCAG-3'
	5'-CTGACCCTTTTTTGGACTTCAGGTGG-3'
M-CSF	5'-CTCCAGCCCGCAGCTCCAGGAGTCTG-3'
	5'-CCCTCTACACTGGCAGTTCCACCTG-3'

In each case, these oligonucleotides were chosen to amplify sequences which span intron splice junctions in order to minimalize the nonspecific signal generated by contaminating genomic DNA (55). In the case of the K-ras oligos bind to sequences within exons II and III, flanking intron 2. In the case of H-ras and N-ras, the oligos bind to sequences within exons III and IV, flanking intron 3. In the case of c-erbB2, the oligos bind to sequences in exons II and IV flanking introns 2 and 3 and amplifying DNA corresponding to exon III. In the case of phl-abl the oligos bind to sequences within exon III f the bcr region and x n II of abl flanking

two introns in the chimeric message and amplifying DNA corresponding to exon I f abl. In the case f p53 the oligos bind to s quences within exons IX and XI and amplify DNA corresponding to exon X. The oligonucleotides for M-CSF are used in control experiments and are complementary to regions of M-CSF exons VI and VIII and amplify the region corresponding to exon VII.

K. High Throughput Quantitative PCR

Current methods for measuring changes in gene expression suffer from various limitations. Conventional direct analysis of changes in mRNA levels (nuclease protection, Northern blot, primer extension) lack sufficient sensitivity for use with high throughput formats (e.g. 96 well plate cell culture). These methods also require difficult analytical procedures (e.g. sequencing gels) complicating automation. The use of gene fusions (luciferase or CAT transcriptional fusions) as demonstrated above, provide sufficient sensitivity and ease of analysis but require disruption of the native transcription unit and loss of chromosomal context, leading to potential artifact. This section of the invention proposes to circumvent the sensitivity limitation of direct analysis by using the amplification potential inherent in the polymerase chain reaction. Combining PCR with the ease of florescence detection of will allow direct mRNA analysis in a high throughput mode.

The following description outlines a high throughput drug screen utilizing direct PCR quantitation of mRNA in its most simple format.

1. Grow cells. Cells are grown in 96 well microtitr plates as described above. The final detection step is a fluorescence measurement, so an opaque (non-reflecting black) plate is required.

2. Add compounds. As with the luciferase reporter screen, compounds are added at several concentrations and at several replications. The number of duplicate samples required can be determined statistically after the basic assay is formatted (currently, quadruplicates are required).

3. Incubate. The incubation time depends on the biology of the systems studied. As with the current luciferase reporter assay, the incubation time is 24 hours.

4. Lyse cells: The cells are lysed in a buffer which satisfies several important criteria: A. avoidance of extremes of temperature; B. complete inactivation of contaminating cellular nucleases; C. compatible with subsequent RNA purification steps; D. rapid and efficient lysis. Chaotropic buffers have been described which satisfy these requirements. Guanidine HCl (6M) will efficiently lyse cells and effectively inactivate cellular nucleases. The kinetics of nucleic acid hybridization are largely unaffected by these conditions. Thus, the subsequent RNA purification (separation using magnetic oligo dT beads, see below) does not require a buffer change.

5. Add external control: The exponential nature of the PCR amplification step tends to magnify small differences in conditions. Key to the usefulness of

this approach is the careful inclusion of appropriate standards for control purposes. An artificial polyadenylated RNA (generated with phage T7 RNA polymerase and commercially available vectors, Promega Madison WI) is added at this point. This RNA serves as a control for all of the following steps: purification, cDNA synthesis, PCR amplification, PCR product purification and detection. This control RNA is added to several lysates at varying concentrations, generating a standard curve.

6. Purify RNA: RNA is purified using commercially available oligo-dT tagged magnetic beads. These beads are added to the lysate, allowed to hybridize to the mRNA, brought to the bottom of the plate using a strong magnet, and extensively washed to remove protein and DNA.

7. Synthesize cDNA: cDNA is generated using the 3' end of the bead bound oligo-dT.

8. Add PCR primers: Each gene (or control) to be assayed requires two oligos. The pair are designed so that they span a large intron. This makes the amplification much more RNA specific. The short, spliced RNA target is much more efficiently amplified than the longer, contaminating genomic DNA target. One of the oligo pair is tagged at its 5' end with a fluorescent label. The other oligo is tagged at its 5' end with biotin (for future purification, see below). Several sets of oligos are added to each lysate. A set for each control and a set for each gene to be assayed. Every set has a different fluorescent tag.

9. PCR Amplification: Simultaneous incubation of many plat s is frequently required, so either a larg array of blocks or a large capacity c nvection oven is necessary.

10. Purification of PCR products: The PCR products are separated from the unreacted oligos using a method similar to the one employed for the initial RNA purification. Magnetic beads, tagged with streptavidan are added to the mixture. PCR products are tagged on one end with biotin (on the other with a fluorescent label) and tightly attach to the magnetic beads. The beads, along with the labeled oligonucleotides, are brought to the bottom of the plate with a magnet and extensively washed. Alternatively, fluorescently tagged PCR products are resolved electrophoretically and quantitated with a scanning gel fluorimeter (Applied Biosystems).

11. Detection: The plates are read in a 96 well fluorimeter (Ammersham).

12. Data analysis: A ratio of fluorescence from a particular gene's PCR product to the signal from the "constitutive" internal control gives the relative mRNA level. Changes in this ratio indicates a change in gene expression. Absolute mRNA levels are determined by control experiments using carefully quantitated artificial RNAs to construct standard curves for each gene studied. This establishes a given ratio (to the internal control) for a given cellular RNA concentration.

L. Isolation of Singl Cell Clon s Containing Various Promoter-Luciferase Fusion Constructs

1. pMluci into NIH3T3 (MMTV control cell line)

pMluci and pSV2Neo, an antibiotic resistance plasmid (48), were co-transfected into NIH/3T3 mouse fibroblast cells using the calcium phosphate precipitation method (56) with a commercially available kit (Pharmacia, Piscataway NJ). Two days later, cells were transferred to media containing 0.4 mg/ml G418 and were grown for an additional 10-14 days. G418-resistant clones were isolated by standard methods. Once sufficient cell numbers were obtained, clones were analyzed based on several criteria: constitutive luciferase production, induction of luciferase expression by dexamethasone (1 μm, Sigma, St. Louis, MO), satisfactory attachment to microtiter plates used in the high-throughput screen (see section G) and acceptable standard deviation in multiple luciferase expression assays (see below for assay protocol). This analysis was carried out using the luciferase assay conditions described above. Of the clones which satisfied the above criteria for the high throughput screen, one clone, M10, was selected for use.

2. neu (pNEU106) into HTB30

75 micrograms of the pNEU106 plasmid was linearized by a single restriction endonuclease cleavage within the vector backbone and electroporated into HTB30 human breast carcinoma cells. Neomycin resistant clones were isolated and tested for luciferase activity. Clones testing positive for luciferase production were subjected to Southern blot analysis (see below). The best clone (pr ducing the highest signal and carrying a single

intact copy of the transfected DNA) was utilized for high throughput screening (designated clone N-2).

3. K-ras (pKRAS106) into SW480

75 micrograms of the pKRAS106 plasmid was linearized by a single restriction endonuclease cleavage within the vector backbone and electroporated into SW480 human breast carcinoma cells. Neomycin resistant clones were isolated and tested for luciferase activity. Clones testing positive for luciferase production were subjected to Southern blot analysis (see below). The best clone (producing the highest signal and carrying a single intact copy of the transfected DNA) was utilized for high throughput screening (designated clone K-2).

4. H-ras (pHRA521) into human colon adenocarcinoma cell line

75 micrograms of the pHRA521 plasmid was linearized by a single restriction endonuclease cleavage within the vector backbone and electroporated into a human colon adenocarcinoma cell line. Neomycin resistant clones were isolated and tested for luciferase activity. Clones testing positive for luciferase production were subjected to Southern blot analysis (see below). The best clone (producing the highest signal and carrying a single intact copy of the transfected DNA) was utilized for high throughput screening (designated clone H21)

6. phl (pPHL106) into K562

75 micrograms of the pPHL106 plasmid was linearized by a single restriction endonuclease cleavage within the vector backbone and electroporated into K562 cells.

N omycin resistant clones were isolated and tested for luciferase activity. Clones testing positive for lucif rase production were subject d to South rn blot analysis (see below). The best clone (producing the highest signal and carrying a single intact copy of the transfected DNA) was utilized for high throughput screening (designated clone bK108).

7. p53 (pP531106 and pP532106) in SW480

75 micrograms of both the pP531106 and pP532106 plasmids are linearized by a single restriction endonuclease cleavage within the vector backbone and separately electroporated into SW480 human colon carcinoma cells. Neomycin resistant clones are isolated and tested for luciferase activity. Clones which test positive for luciferase production are subjected to Southern blot analysis (see below). The best clones (producing the highest signal and carrying a single intact copy of the transfected DNA) representing each plasmid are utilized for high throughput screening (designated clones P531 and P532).

8. pCM106 into Hep3B (a control cell line)

Hep3B hepatocellular carcinoma cells were transfected by electroporation with 75 micrograms of pCM106 which had been linearized by a single ScaI cut within the vector backbone. Neomycin resistant colonies were isolated and tested for luciferase activity. Luciferase positive, neomycin resistant clones were subjected to Southern blot analysis (see below). The best clone, producing the most luciferase activity from a single, correctly integrated vector was selected for use as the CMV reporter cell line in the HTP scre n (this cl ne was d signated CM1).

M. High-Throughput (HTP) Screening

Cell plating: Dynatech Microlit r 96 well plates were custom pretreated for cell attachment by Dynatech Laboratories, Inc. (Chantilly, VA). Alternatively, the 96 well plates were treated with 50 µl per well of human fibronectin (hFN, 15 µg/ml in PBS, Collaborative Research, Bedford, MA) overnight at 37°C. hFN-treated plates were washed with PBS using an Ultrawash 2 Microplate Washer (Dynatech Labs), to remove excess hFN prior to cell plating. N-2 (the neu reporter) K-2 (the K-ras reporter) and CM1 (the CMV reporter, added as a control) maintained in their respective serum media (with 0.2 mg/ml G418) were washed with PBS, harvested by trypsinization, and counted using a hemocytometer and the Trypan Blue exclusion method according to protocols provided by Sigma, St. Louis, MO Chemical Company. Cells were then diluted into media (with 0.2 mg/ml G418), and 0.2 ml of cell suspension per well was plated using a Millipore MultiDrop Dispenser (Millipore Corp., Bedford, MA). Plates were incubated overnight at 37°C in a humidified 5% CO_2 atmosphere. Chemicals from the chemical file of one of Oncogene Science's corporate partners were dissolved in DMSO at concentrations of 3-30 mg/ml. The next day a fully automated device, as described in U.S. patent application #382,483, was used to incubate luciferase reporter cells in 96-well microtiter plates, transfer chemicals and known transcriptional modulators to the cells, incubate cells with the chemicals, remove the chemicals by washing with PBS, add lysis buffer to the cells and measure the bioluminescence produced. The cell lysis buffer was modified to also contain the luciferin. Therefore, lysis of cells and the bioluminescence reaction begin simultaneously and the production of bioluminescent light reaches a maximum at

about 5 min. The level of light output d clines by about 20% within furth r 30 min. For better lysis buffer stability bovin serum albumin has been omitted. This improved lysis buffer has been shown to remain fully functional for at least 12 hours, when kept on ice and protected from direct light.

RESULTS

A. In vivo signal half-life of the luciferase reporter system

When screening for inhibitors rather than inducers of transcription, the half-life of the reporter molecule becomes a crucial parameter in determining the minimal incubation time that would be necessary to allow enough decay of reporter molecules so that the inhibition of their synthesis became visible. The oncogene reporter cell line were therefore tested for the time dependency of luciferase activity after treatment of the cells with Actinomycin D, an inhibitor of transcription. This experiment measured the combined half-life of luciferase mRNA and of the luciferase protein and compares the rate of signal decay of the H-ras, K-ras and c-erbB2 reporter cell lines to a CMV reporter cell line control. Cells derived from clones CM1 (CMV), K-2 (K-ras), H21 (H-ras) and N-2 (c-erbB2) were seeded into 96-well microtiter plates and incubated overnight. At time 0, Actinomycin D (25 μg/ml) was added. At the times indicated in Figure 26, cells were washed with PBS and luciferase activity of Actinomycin-treated cells determined as described in Materials and Methods. The signal from the treated cells was compared to the luciferase activity of untreated controls. The logarithm of the treated/untreated ratio was plotted versus time, this data is shown in Figure 26. The calculated half-life of the signal from each of the four cell lines is shown in table 1. The half-lives were found to range from about 3 to 10 hours indicating that a 24 hour incubation with a 100% efficient inhibitor of transcription would be sufficient to reduce luciferase levels to 6% of the control in the tested cell lines.

TABLE 1
Half-life Determinations

Cell Line	Reporter	Signal Half-life
CM1	CMV	6.5 hours
K2	K-ras	6.5 hours
H21	H-ras	10 hours
N2	neu	3 hours

B. Southern Blot

An autoradiogram from a typical Southern blot analysis is shown in Figure 27. Genomic DNA from five neomycin resistant, luciferase producing clones, transfected with the H-ras reporter vector pHRAS106 was digested with DraI, blotted and probed as described in Materials and Methods, along with two control samples; one consisting only of genomic DNA from the untransfected host strain (SW480) and the other consisting of a mixture of SW480 genomic DNA and DraI digested pHRAS106 (to provide a size control for the expected band. In this case all five clone produced the expected band (and no other) demonstrating that the transfected vector had integrated into the host cell genome with out undergoing gross rearrangement. In other transfections, correctly integrated vectors represented between 100% and 20% of the neomycin resistant, luciferase producing clones.

C. PCR Detection and Quantitation

To test the quantitative ability of the polymerase chain reaction, total RNA was isolated form U5637 bladder carcinoma cells and diluted in two-fold serial steps to

yield samples ranging from 4 to 0.05 μg. These RNA samples were used to generate cDNA using random primers (55) and then mixed with a constant amount of phage lambda DNA (0.2 ng) as a control for amplification efficiency. Alpha-32P-dATP was included in the PCR buffer to allow quantitation of the amplified products. The M-CSF specific PCR oligonucleotides describe above were added to the standard reaction mixture (2 pmoles per sample) and PCR carried out for 35 cycles in a Perkin-Elmer-Cetus thermal cycler. The products of the reaction were electrophoresed on a 3% NuSieve agarose gel. The gel was dried and used to expose Kodak X-OMAT AR film. The resulting autoradiogram is shown in Figure 28. This autoradiogram was quantitated using an LKB laser densitometer, the data are shown in Figure 29. The graph plots the amount of M-CSF specific product divided by the constant lambda DNA signal. The reaction was clearly quantitative for the RNA samples between 0.05 and 1 μg (total RNA), and proved to be a very sensitive assay for M-CSF mRNA, which is barely detectable in this cell line by conventional S1 analysis.

To test the specificity of the PCR reaction, the oligos for detection of c-*erb*B2, N-*ras*, H-*ras* and K-*ras* were used to amplify cDNA derived from 10 and 0.4 ng of U5637 total RNA. The data are shown in Figure 30. In each sample a sample of phage lambda DNA and lambda control oligos was added to control for the efficiency of the PCR reaction. This is the uppermost, consistent band in each lane. In each case the oligos specifically amplifies a DNA fragment of the correct molecular weight. A few extra bands of unknown origin appear in the c-*erb*B2 lanes and in the lane representing the highest concentration of H-*ras* mRNA.

D. Quality Assuranc Analysis

A number of quality assuranc criteria are routinely assessed during the course of high throughput screens. Data from QA analysis of a portion of Screen III are shown in Figures 31-34. Figure 31 shows an analysis of the consistency of the luciferase signal on various areas of each plate. The ratios of negative control values from three different areas within each plate are calculated and plotted versus plate number. The expected value is 1.0. Values greater than 1.5 or less than 0.4 indicate uneven signal generation across the plate. In this example 240 plates, representing 1440 compounds, tested against three cell lines, are shown. The coefficient of variance for the 12 negative control values from each of the same 240 plates are represented by the data shown in Figure 32. Values less than 20% are considered acceptable. Similar data for the 12 positive control values of the same plates are shown in Figure 33. Figure 34 shows the transcription induction ratio (TIR) for the positive controls of one cell line represented in the same set of 240 plates. The TIR is the ratio of the experimental values to the untreated controls. In this case the cell line is the K-ras reporter and the positive control is Actinomycin D a potent general inhibitor of transcription. Three values are shown for each plot, representing three different concentrations of Actinomycin D. The expected value for such an analysis depends on the half life of the signal and the incubation time (here 24 hours), but for this combination, typical values range from 0.4 to 0.3 fold.

E. High-Throughput Drug Screen

Table 2 presents the data from a three week high throughput screen of 2334 compounds. Three cell lines were utilized; CM1 (the CMV reporter cell line) as a control for nonspecific effects. N-2 (the c-erbB2 reporter cell line) and K-2 (the K-ras reporter cell line). Each compound was assayed at three concentrations in quadruplicate. Each microtiter plate included a negative control row (no added compound) and a positive control row (Actinomycin D at three concentrations). The data are reported as TIR (transcription induction ratio) which is the median of the samples quadruplicate values divided by the median of the negative control values. In this case transcriptional inhibitors are sought, so the selection criteria for lead compounds is that the test promoter be inhibited to 0.4 of the negative control while the other cell lines remain within 0.8X of the control value. During these three weeks 10 compounds scored positive for the specific inhibition of the K-ras promoter, 19 scored as leads for the inhibition of the c-erbB2 promoter and 39 compounds inhibited nonspecifically. Compounds scoring as leads in the primary screen are repeated and then subjected to secondary analysis such as effects on the minigene transfectant phenotypes (see above).

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Oncogene, Science Inc.

(ii) TITLE OF INVENTION: Methods of Transcriptionally Modulating Expression of Oncogenes and Tumor Suppressor Genes

(iii) NUMBER OF SEQUENCES: 34

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 17-JAN-1992
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGGATCGCAG CGCTGCCTTT CCT
23

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CATGAGGAAA GGCAGCGCTG CGATCCAGCA C
31

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGGCGCAGCG CTCCAGGAGA AGCTG
25

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGCTATGGAG TTGGCTCAAG CAGCCTGC
28

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGCGGGTCTG TAGGCAGGTC GGCTC
25

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CAGTAAGAGC TCAGCCCTTG CCCTGGGCAG G
31

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTCCAGCCCG CAGCTCCAGG AGTCTG
26

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCCTCTACAC TGGCAGTTCC ACCTG
25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGCCAAGGAG GCCGAGAATA TCACG
25

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCCAGACTTC TACGGCCTGC TGCCCGAC
28

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TCAGCAATTG AGAGCATTCT TAAA
24

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTCCTTGATA TGGATTGGAT GTCG
24

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ACTAATAATG TAAAAGACGT CACTAAATTG

30

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TCTCGCTTAT CCAACAATGA CTTGG
25

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CCAGAACAGC TAAACGGAGT CGCCACACCA CTGTTTGTGC
40

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CATGGGGCCG GAGCCGCAGT GAGCAC
26

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 bas pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CATGGTGCTC ACTGCGGCTC CGGCCC
26

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TCGAGATCTG AGGCCTGCTG ACCATGGGGG CC
32

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CCCATGGTCA GCAGGCCTCA GATC
24

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 bas pairs
(B) TYPE: nucl ic acid
(C) STRANDEDNESS: singl
(D) TOPOLOGY: lin ar

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CTCTTGGATA TTCTCGACAC ACAGCA
26

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GTGTCTACTG TTCTAGAAGG
20

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GCACGCACTG TGGAATCTCG G
21

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 bas pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

ACGTCGAGTA CGTCGGCCCC GGTG
24

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GCCCACGAAC TGGCCAAGAG TT
22

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CTGAGTCCCA TCATCACTGC TCTT
24

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 bas pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCAATGCCAG CCTGTCCTTC CTGCAG
26

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GATCAAGACC CCTCCTTTCA AGATCT
26

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AATTCCGCTG ACCATCAATA AGGAAG
26

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 bas pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: lin ar

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

ATAGCCTAAG ACCCGGAGCT TTTCAC
26

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GATGGAGAAT ATTTCACCCT TCAG
24

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CTGACCCTTT TTGGACTTCA GGTGG
25

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: lin ar

(ii) MOLECULE TYPE: DNA (g n mic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CTCCAGCCCG CAGCTCCAGG AGTCTG
26

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CTCCAGCCCG CAGCTCCAGG AGTCTG
26

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CCCTCTACAC TGGCAGTTCC ACCTG
25

What is claimed is:

1. A method of directly transcriptionally modulating the expression of an oncogene or tumor suppressor gene, the expression of which is associated with a defined physiological or pathological effect within a multicellular organism, which comprises contacting a cell, which is capable of expressing the gene, with a molecule at a concentration effective to transcriptionally modulate expression of the gene and thereby affect the level of the oncogene or tumor suppressor gene product(s) encoded by the gene which is expressed by the cell, which molecule (a) does not naturally occur in the cell, (b) specifically transcriptionally modulates expression of the oncogene or tumor suppressor gene, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

2. A method of claim 1, wherein the molecule does not naturally occur in any cell of a lower eucaryotic organism.

3. A method of claim 1, wherein the molecule does not naturally occur in any cell.

4. A method of claim 1, wherein the molecule is not a naturally occurring molecule.

5. A m thod of claim 1, wher in th cell is a cell of

th multicellular rganism.

6. A method of claim 1, wherein the cell is an animal cell.

7. A method of claim 6, wherein the animal cell is a human cell.

8. A method of claim 1, wherein the transcriptional modulation comprises upregulation of expression of the oncogene or tumor suppressor gene.

9. A method of claim 1, wherein the transcriptional modulation comprises downregulation of expression of the oncogene or tumor suppressor gene.

10. A method of claim 1, wherein the molecule binds to a modulatable transcription sequence of the gene.

11. A method of claims 1, wherein the molecule comprises an antisense nucleic acid.

12. A method of claim 1, wherein the molecule comprises double-stranded nucleic acid.

13. A method of claim 1 wherein the molecule comprises a nucleic acid capable of forming a triple helix with double-stranded DNA.

14. A method of claim 1, wherein the gene is an oncogene.

15. A method of claim 14, wherein the oncogene is a c-neu gene.

16. A method of claim 14, wherein the oncogene is a c-K-ras gene.

17. A method of claim 14, wherein the oncogene is a c-H-ras gene.

18. A method of claim 14, wherein the oncogene is a c-N-ras gene.

19. A method of claim 14, wherein the oncogene is a phl-abl gene fusion.

20. A method of claim 14, wherein the oncogene is a myc gene.

21. A method of claim 14, wherein the oncogene is a fms gene.

22. A method of claim 14, wherein the oncogene is a erbA gene.

23. A method of claim 14, wherein the oncogene is a p53 gene.

24. A method of claim 14, wherein the oncogene is a retroviral oncogene.

25. A method of claim 1, wherein the gene is a tumor suppressor gene.

26. A method of claim 25 wherein the tumor suppressor gene is a p53 gene.

27. A method of claim 25 wherein the tumor suppressor gene is a retinoblastoma gene.

28. A method of claim 25 wh rein the tumor suppressor gene is a Wilms' tumor gene.

29. A method of claim 25 wherein the tumor suppressor gene is a neurofibromatosis type 1 gene.

30. A method of claim 25 wherein the tumor suppressor gene is a DCC gene.

31. A method of claim 25 wherein the tumor suppressor gene is an erbA gene.

32. A method of claim 25 wherein the tumor suppressor gene is an adenomatous polyposis coli gene.

33. A method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of an oncogene or tumor suppressor gene, which comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the oncogene or tumor suppressor gene, (ii) a promoter of the oncogene or tumor suppressor gene, and (iii) a DNA sequence encoding a polypeptide other than the oncogene or tumor suppressor, which polypeptide being capable of producing a detectable signal, which DNA sequence is coupled to, and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the oncogene or tumor suppressor gene, causes a measurable detectable signal to be produced by the

polypeptide so expressed, quantitatively determining the amount of the signal produced, comparing th amount so determined with the amount of produced signal detected in the absence of any molecule being tested or upon contacting the sample with any other molecule, and thereby identifying the molecule as one which causes a change in the detectable signal produced by the polypeptide so expressed, and thus identifying the molecule as a molecule capable of transcriptionally modulating the expression of the oncogene or tumor suppressor gene.

34. A method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of an oncogene or tumor suppressor gene, which comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the oncogene or tumor suppressor, (ii) a promoter of the oncogene or tumor suppressor gene, and (iii) a reporter gene, which expresses a polypeptide, coupled to, and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the oncogene or tumor suppressor gene, causes a measurable change in the amount of the polypeptide produced, quantitatively determining the amount of the polypeptide so produced, comparing the amount so determined with the amount of polypeptide produced in the absence of any molecule being tested or upon contacting the sample with any other mol cule, and ther by

identifying the molecule as ne which causes a change in the amount of th polypeptid expressed, and thus identifying the molecule as a molecule capable of transcriptionally modulating the expression of the oncogene or tumor suppressor gene.

35. A method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of an oncogene or tumor suppressor gene, which comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the oncogene or tumor suppressor gene, (ii) a promoter of the oncogene or tumor suppressor gene, and (iii) a DNA sequence transcribable into mRNA coupled to and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the oncogene or tumor suppressor gene, causes a measurable difference in the amount of mRNA transcribed from the DNA sequence, quantitatively determining the amount of the mRNA produced, comparing the amount so determined with the amount of mRNA detected in the absence of any molecule being tested or upon contacting the sample with any other molecule, and thereby identifying the molecule as one which causes a change in the detectable mRNA amount of, and thus identifying the molecule as a molecule capable of transcriptionally modulating the expression of the oncogene or tumor suppressor gene.

36. A method of claim 33, 34 or 35, wherein the sampl

compris s cells in mon layers.

37. A method of claim 33, 34, or 35, wh rein the sample comprises cells in suspension.

38. A method of claim 33, 34 or 35, wherein the cells comprise animal cells.

39. A method of claim 38, where the animal cells are human cells.

40. A method of claim 33, 34 or 35, wherein the predefined number of cells is from about 1 to about 5×10^5 cells.

41. A method of claim 40, wherein the predefined number of cells is from about 2×10^2 to about 5×10^4 cells.

42. A method of claim 33, 34 or 35, wherein the predetermined amount of the molecule to be tested is based upon the volume of the sample.

43. A method of claim 33, 34 or 35, wherein the predetermined amount is from about 1.0 pM to about 20 μM.

44. A method of claim 33, 34 or 35, wherein the predetermined amount is from about 10 nM to about 500 μM.

45. A method of claim 33, 34 or 35, wherein the contacting is effected from about 1 to about 24 hours.

46. A method of claim 45, wherein the contacting is eff cted from ab ut 2 to about 12 hours.

47. A method of claim 33, 34 or 35, wherein the contacting is effected with more than one predetermined amount of the molecule to be tested.

48. A method of claim 33, 34 or 35, wherein the molecule to be tested is a purified molecule.

49. A method of claim 33, 34 or 35, wherein the modulatable transcriptional regulatory sequence comprises a cloned genomic regulatory sequence.

50. A method of claim 33, 34 or 35, wherein the DNA consists essentially of more than one modulatable transcriptional regulatory sequence.

51. A method of claim 33 or 34, wherein the DNA sequence encoding the polypeptide is inserted downstream of the promoter of the oncogene or tumor suppressor gene by homologous recombination.

52. A method of claim 33, wherein the polypeptide is a luciferase.

53. A method of claim 33, wherein the polypeptide is chloramphenicol acetyltransferase.

54. A method of claim 33, wherein the polypeptide is β glucuronidase.

55. A method of claim 33, wherein the polypeptide is β galactosidase.

56. A method of claim 33, wherein the polypeptide is n omycin ph sphotransferase.

57. A method of claim 33, wherein the polypeptide is alkaline phosphatase.

58. A method of claim 33, wherein the polypeptide is guanine xanthine phosphoribosyltransferase.

59. A method of claim 34, wherein the polypeptide is capable of complexing with an antibody.

60. A method of claim 34, wherein the polypeptide is capable of complexing with biotin.

61. A method of claim 35, wherein mRNA is detected by quantitative polymerase chain reaction.

62. A screening method of claim 33, 34, 35 or 39 which comprises separately contacting each of a plurality of substantially identical samples, each sample containing a predefined number of cells under conditions such that contacting is affected with a predetermined amount of each different molecule to be tested.

63. A screening method of claim 62, wherein the plurality of samples comprises more that about 10^4 samples.

64. A screening method of claim 62, wherein the plurality of samples comprises more than about 5 X 10^4 samples.

65. A method of ess ntially simultaneously screening

molecules to determine whether the molecules are capable of transcriptionally modulating one or more oncogenes or tumor suppressor genes which comprises essentially simultaneously screening the molecules against the oncogenes or tumor suppressor genes according to the method of claim 62.

66. A screening method of claim 64 or 65, where more than about 10^3 samples per week are contacted with different molecules.

67. A method for directly transcriptionally modulating in a multicellular organism the expression of a oncogene or tumor suppressor gene, the expression of which is associated with a defined physiological or pathological effect in the organism, which comprises administering to the organism a molecule at a concentration effective to transcriptionally modulate expression of the gene and thus affect the defined physiological or pathological effect, which molecule (a) does not naturally occur in the organism and (b) specifically transcriptionally modulates expression of the gene encoding an oncogene or tumor suppressor gene product(s), and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

68. A method of claim 67, wherein the molecule binds to a modulatable transcription sequence of the gene.

69. A method of claim 67, wherein the molecule comprises

an antisense nucleic acid.

70. A method of claim 67, wherein the mol cule comprises a double-stranded nucleic acid molecule.

71. A method of claim 67, wherein the molecule comprises a nucleic acid capable of forming a triple helix with double-stranded DNA.

72. A method of claim 67, wherein the multicellular organism is a human being.

73. A method of claim 67, wherein the multicellular organism is an animal.

74. A method of claim 72, wherein the physiological effect is the protection of non-neoplastic cells from damage by chemotherapeutic agents.

75. A method of claim 72, wherein the defined pathological effect is a disorder and modulated expression of an oncogene or tumor suppressor gene is associated with amelioration of the disorder.

76. A method of claim 72, wherein the defined pathological effect is cancer.

77. A method of claim 72, wherein the defined pathological effect is leukemia.

78. A method of claim 72, wherein the defined pathological effect is malignant melanoma.

79. A method of claim 72, wherein the defined pathological effect is lung canc r.

80. A method of claim 72, wherein the defined pathological effect is colon cancer.

81. A method of claim 72, wherein the defined pathological effect is pancreatic cancer.

82. A method of claim 72, wherein the defined pathological effect is ovarian cancer.

83. A method of claim 72, wherein the defined pathological effect is breast cancer.

84. A method of claim 67 or 72, wherein the administering comprises topical contact.

85. A method of claim 67 or 72, wherein the administering comprises oral, transdermal, intravenous, intramuscular or subcutaneous administration.

Figure 1.
Features of the Mammalian Vector pUV102 with Inserted TK-NEO Cassette.

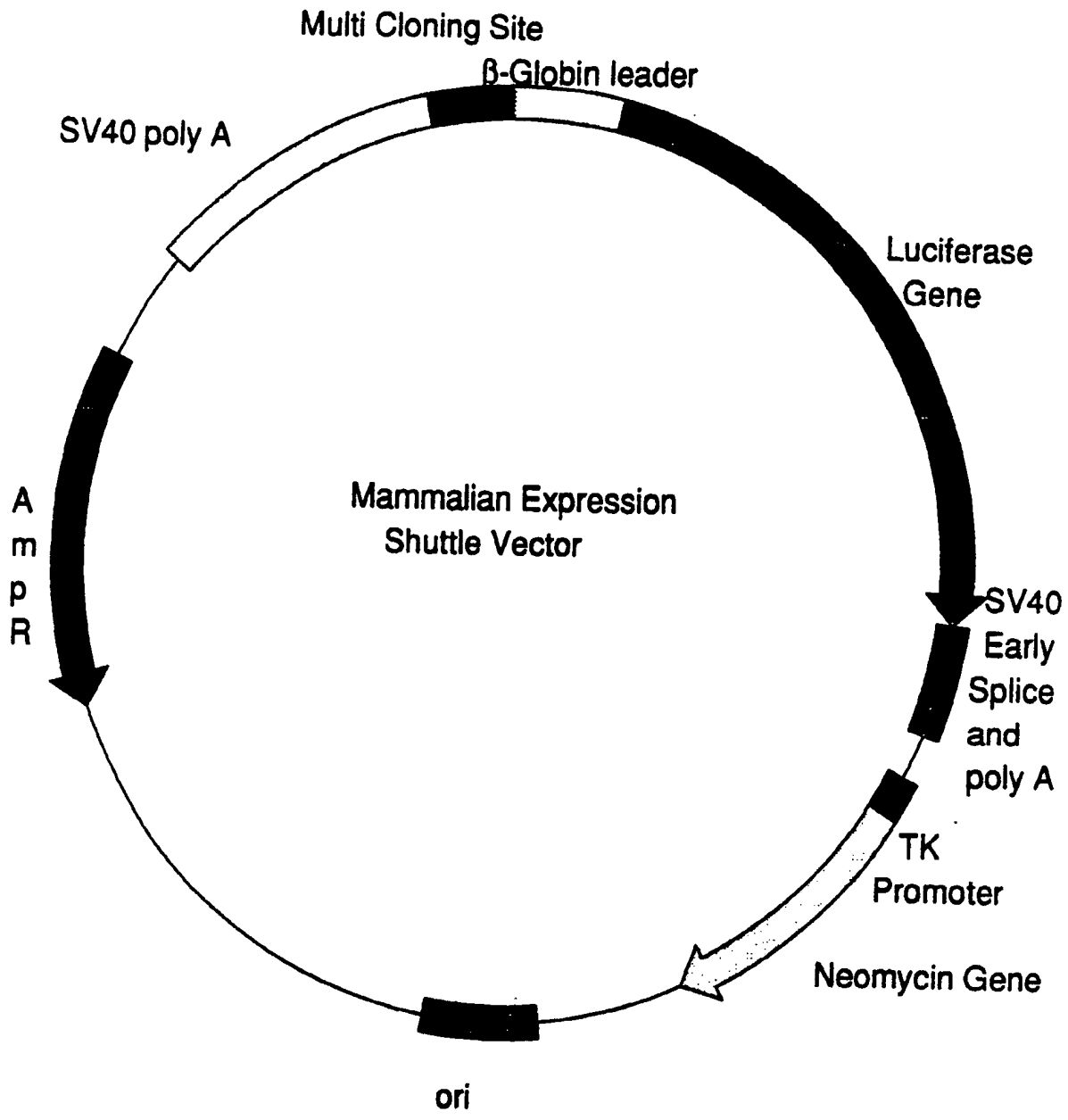


Figure 2.
pDO432.

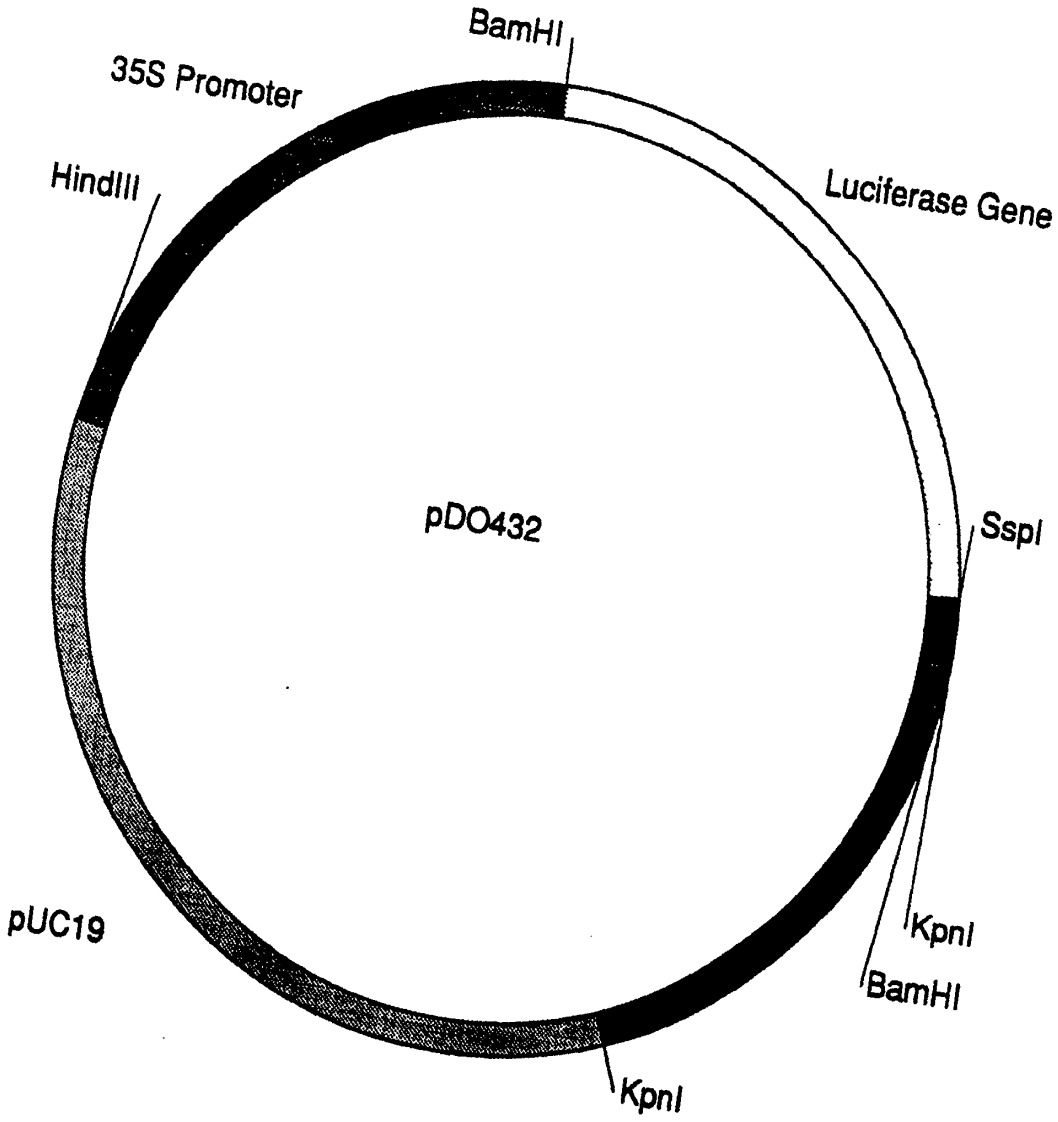


Figure 3.
pSVLuci.

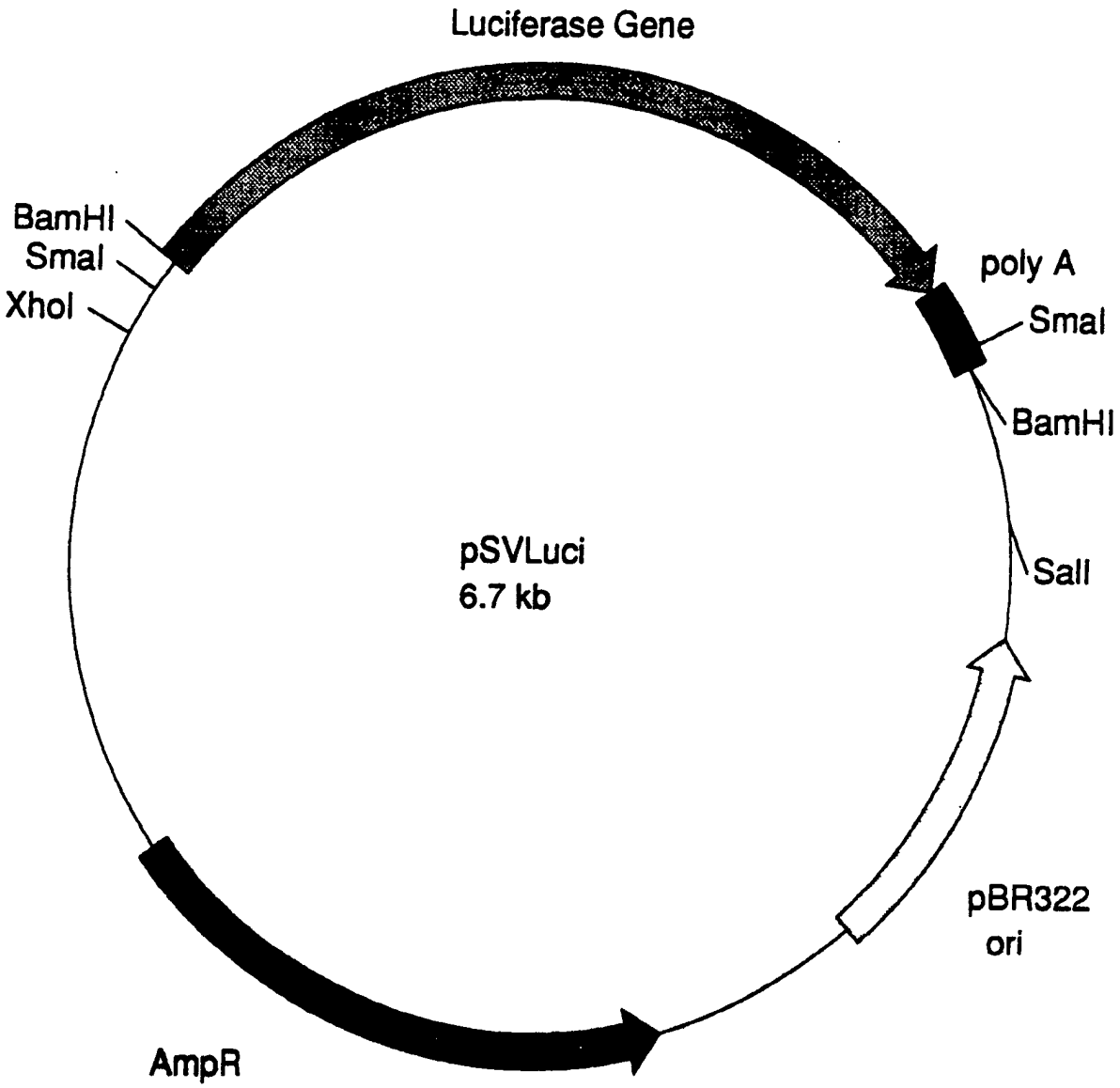


Figure 4.
pMLuci.

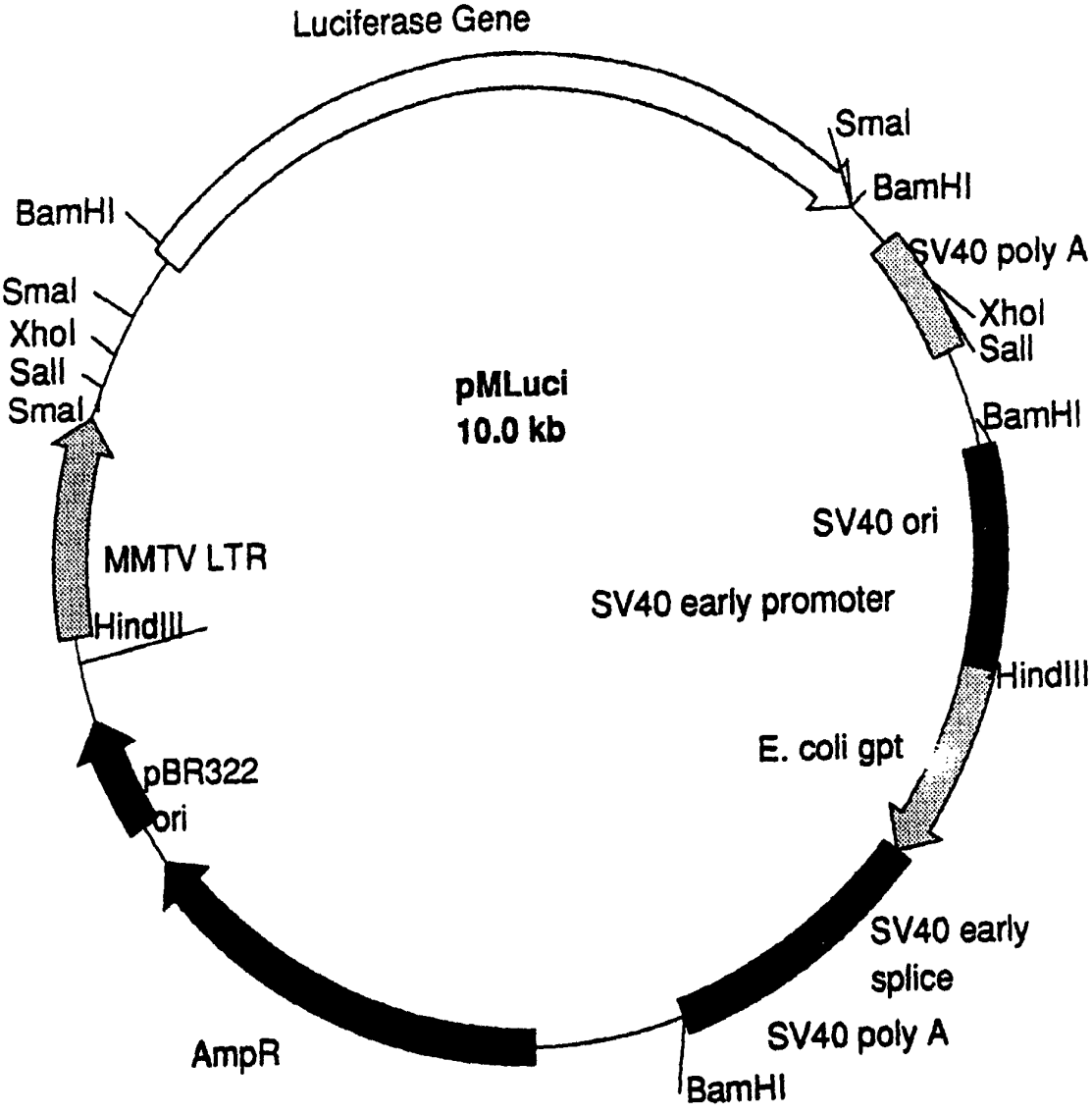


Figure 5.
Sequence of the pUV oligonucleotides.

pUV1:
5'TCGACCCGGGCGGCCGCTGATCAGACGTCGGGCCCGGTACCGTGCACTACGTAAGATCTAA
GCTT3'

pUV2:
5'ACTAGTCTGCAGGCTAGCACTCTTCTGGTCCCCACAGACTCAGAGAGAACCCACCATGGA
3'

pUV3:
5'AGACGCCAAAAACATCAAGAAAGGCCCGGCGCCATTCTATCCTCTAGAGGGGATCCAGC
TG3'

pUV4:
5'TAGATCTTACGTAGTGCACGGTACCGGGCCCGACGTCTGATCAGCGGCCGCCCCGGG3'

pUV5:
5'GGTGGGTTCTCTCTGAGTCTGTGGGGACCAGAAGAGTGCTAGCCTGCGACTAGTAAGCT3'

pUV6:
5'AATTCAGCTGGATCCCCTCTAGAGGATAGAATGGCGCCGGGCCTTTCTTGATGTTTTTGGCGT
CTTCCAT3'

Figure 6

Constructi n f pUV001

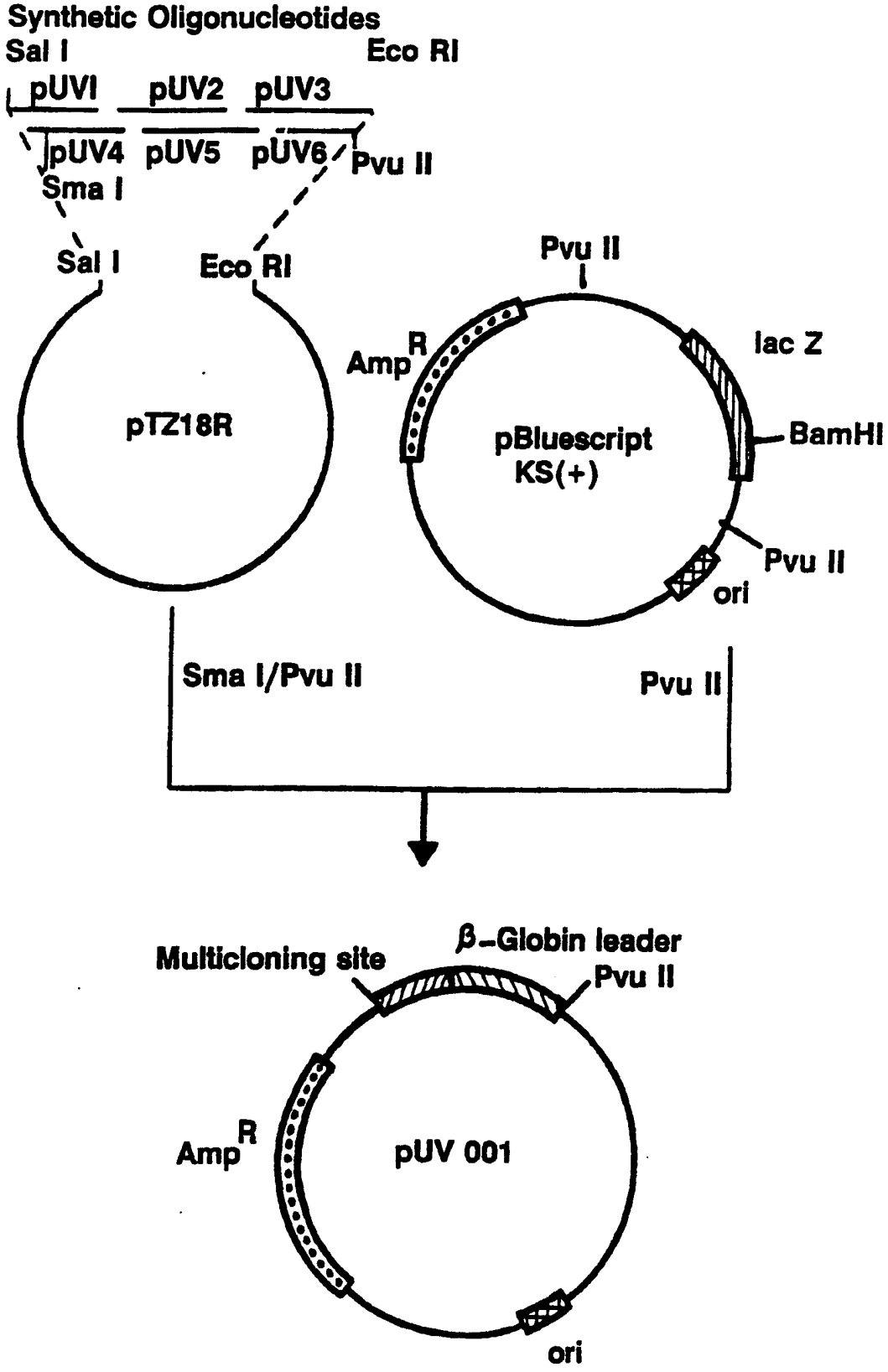
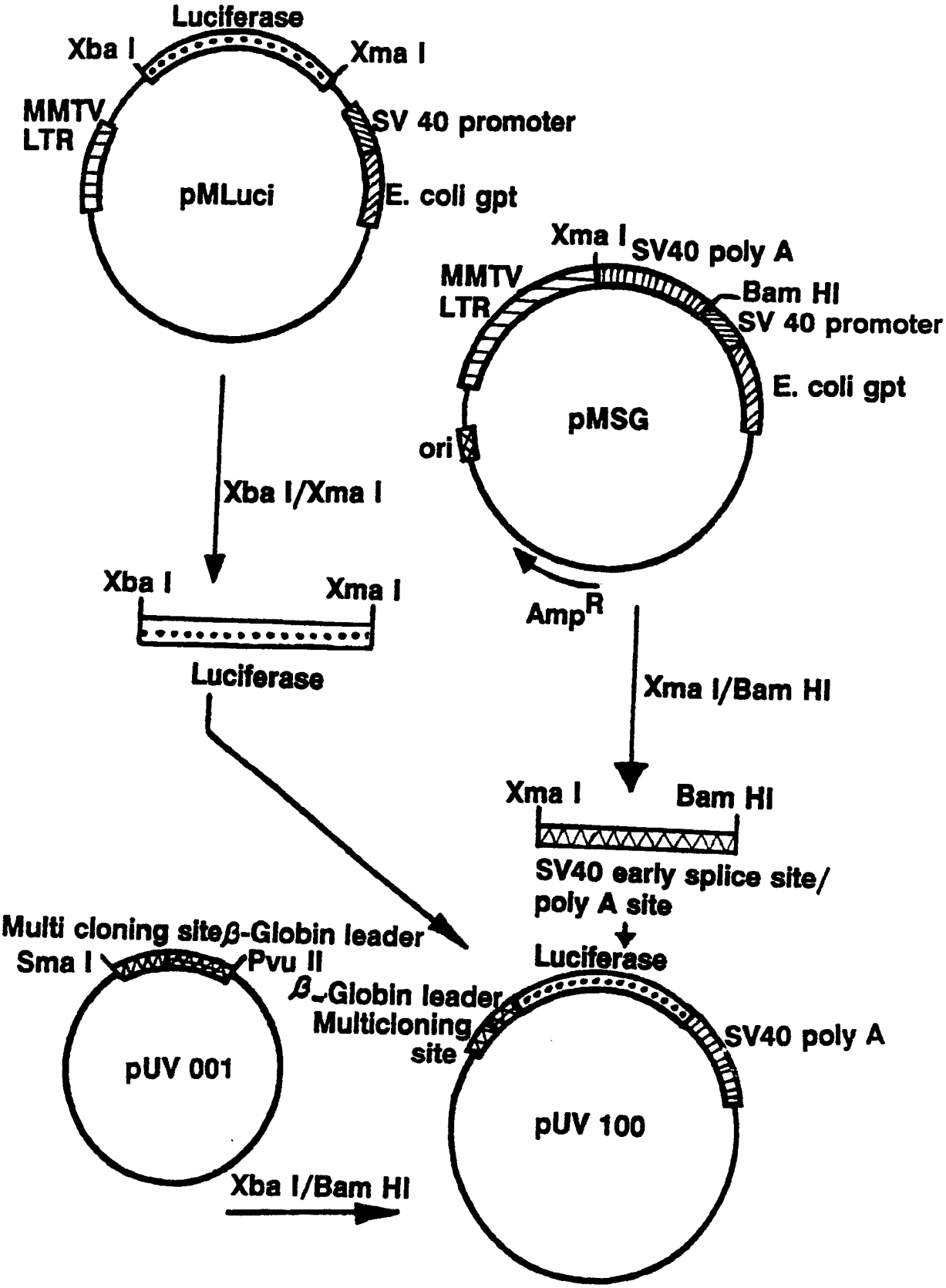
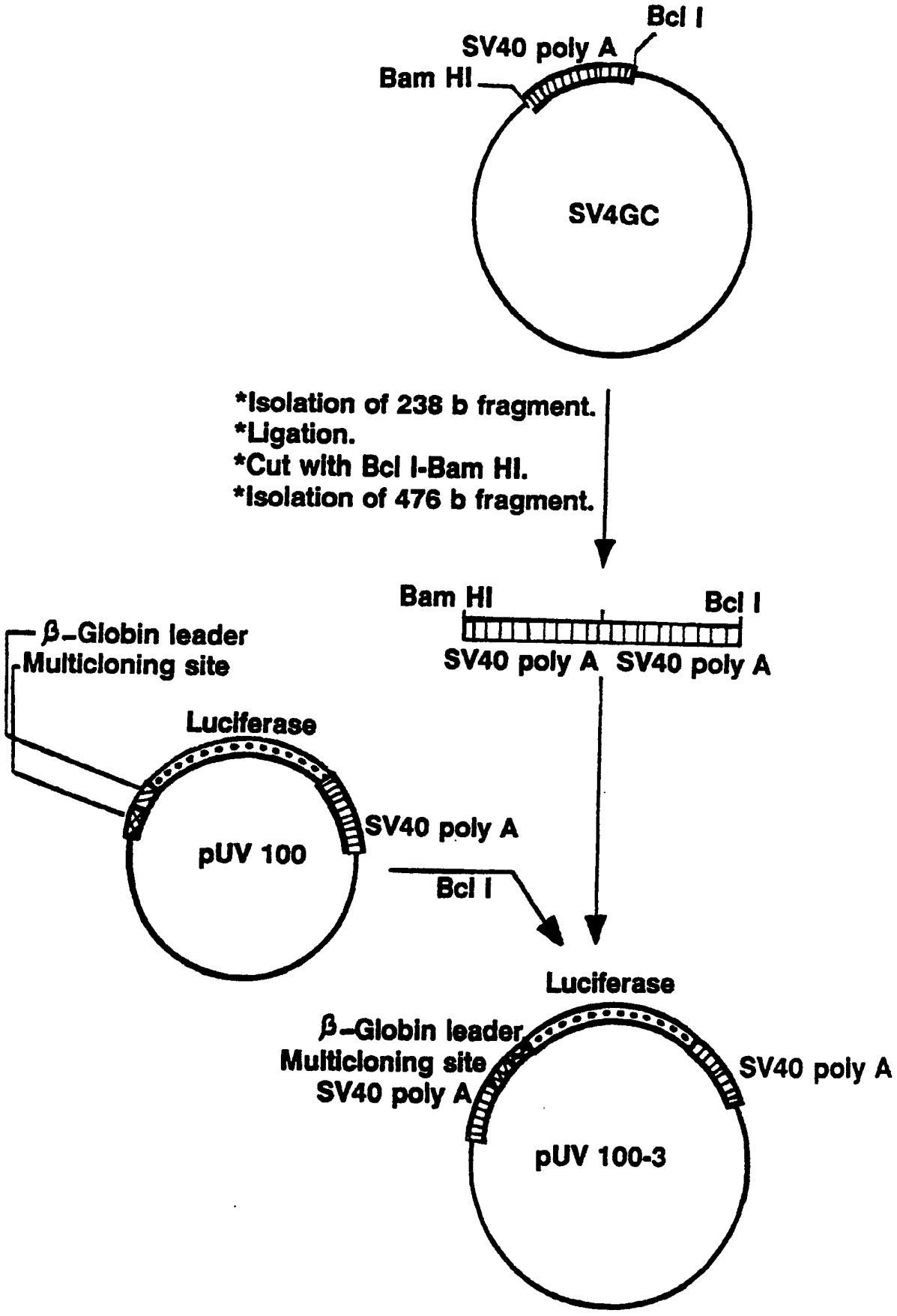


Figure 7
Construction f pUV100



Figur 8
Constructi n of pUV100-3



Figur 9

Construction of pUV102 and pUV103

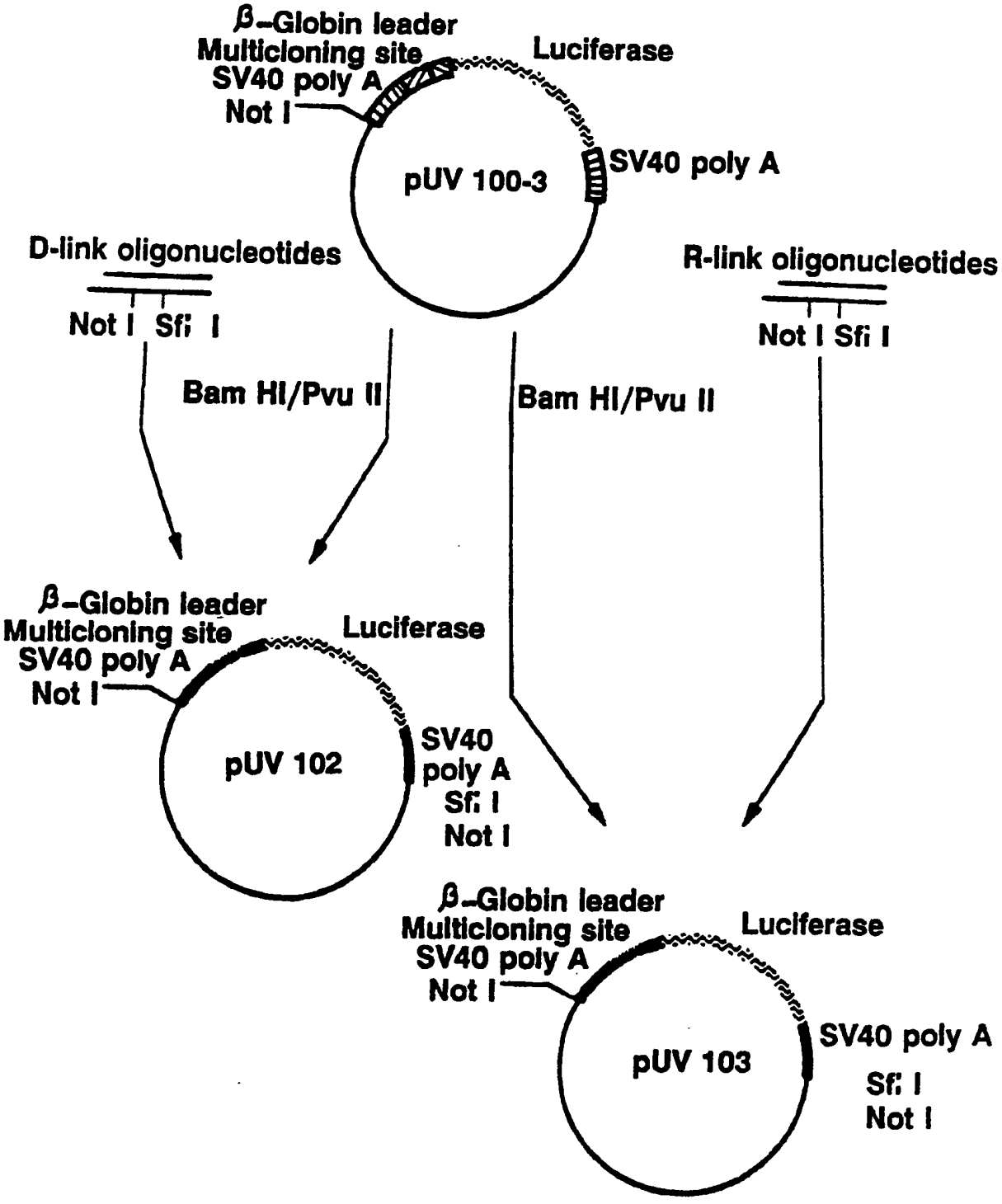


Figure 10

Synthetic HSV-TK Promoter

Oligo #1: 5' AGCTTGGCCCCTAGGGCCACTAGTCTGCAGCTATGATGACACAA
ACCCCGCCCAGCGTCTTGTCATTGGCGA-3'

Oligo #2: 3' ACCGGGGATCCCGGTGATCAGACTCGATACTACTGTGTTTGGGG
CGGGTCGCAGAACAGTAACCGCTTAAGCT-5'

Oligo #3: 5' ATTCGAACACGCAGATGCAGTCGGGGCGGCGCGGTCCGAGGTC
CACTTCGCATATTAAGGTGACGCGTGTGGG-3'

Oligo #4: 3' TGTGCGTCTACGTCAGCCCCGCCGCGCCAGGCTCCAGGTGAAG
CGTATAATTCCACTGCGCACACCCGATC-5'

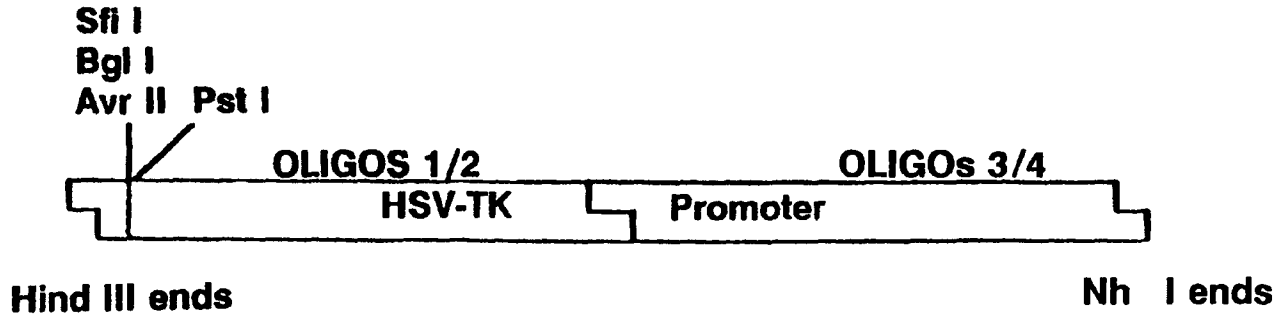


Figure 11

Constuction of pTKL100

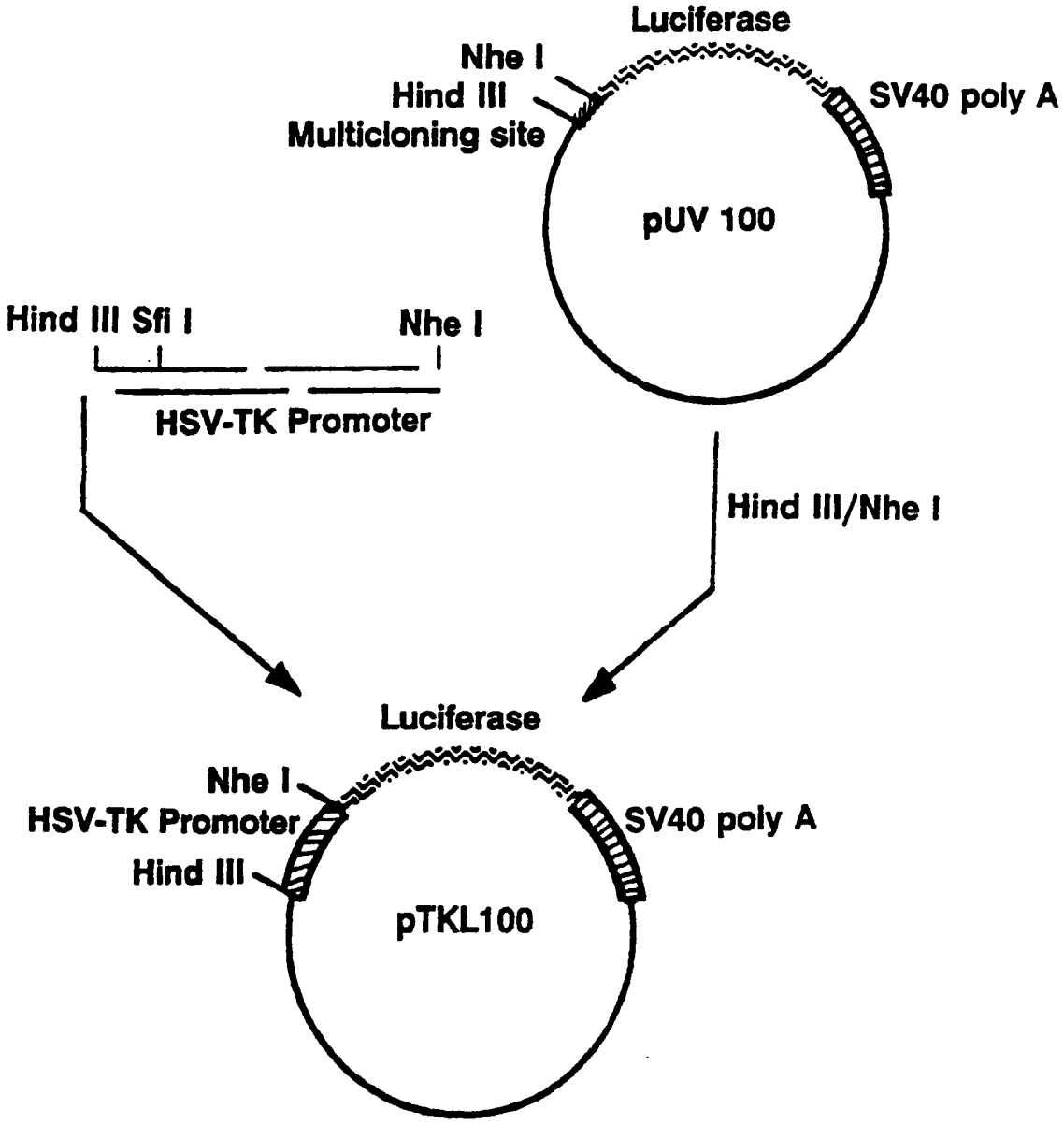


Figure 12

Construction of pTKNEO

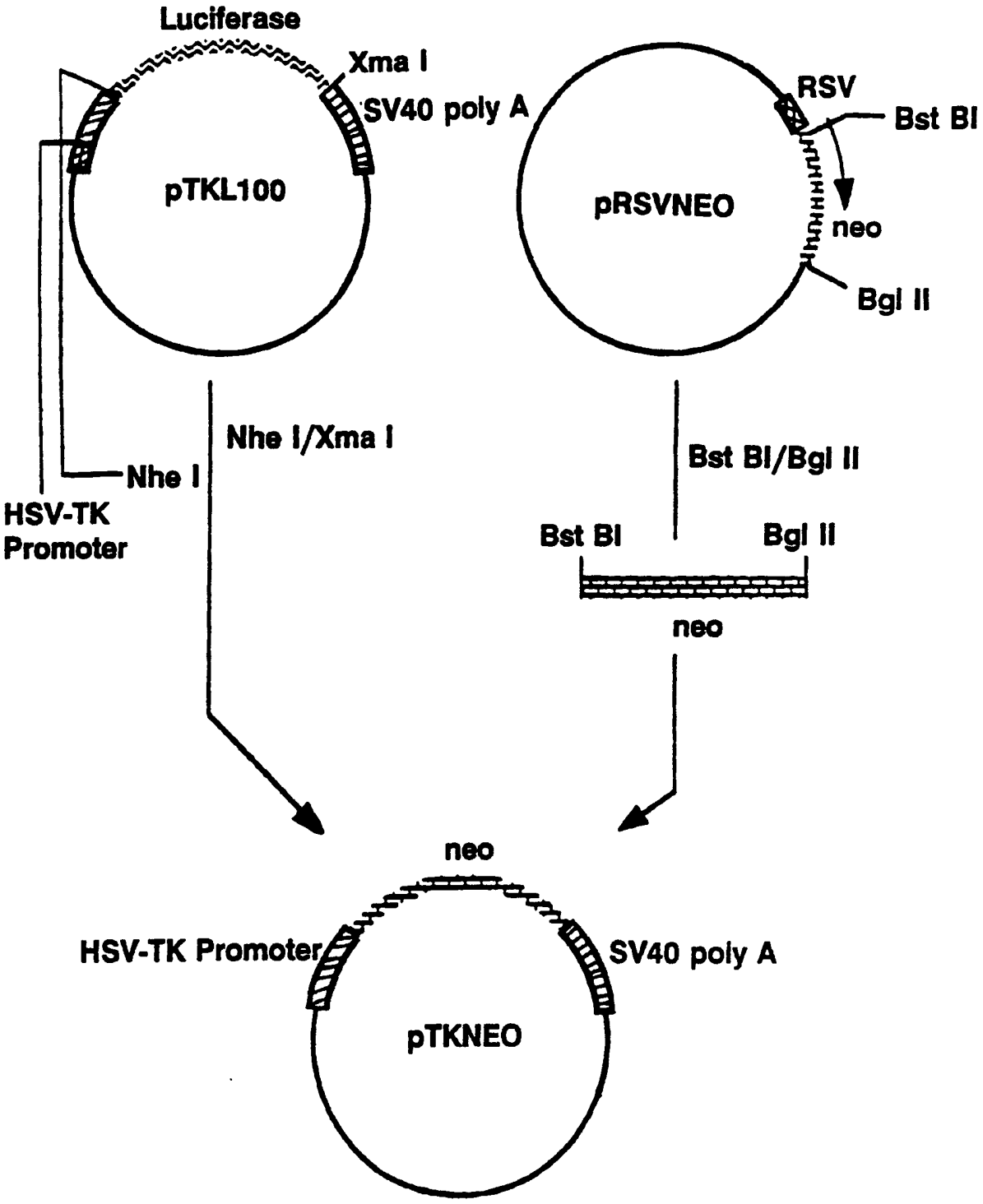
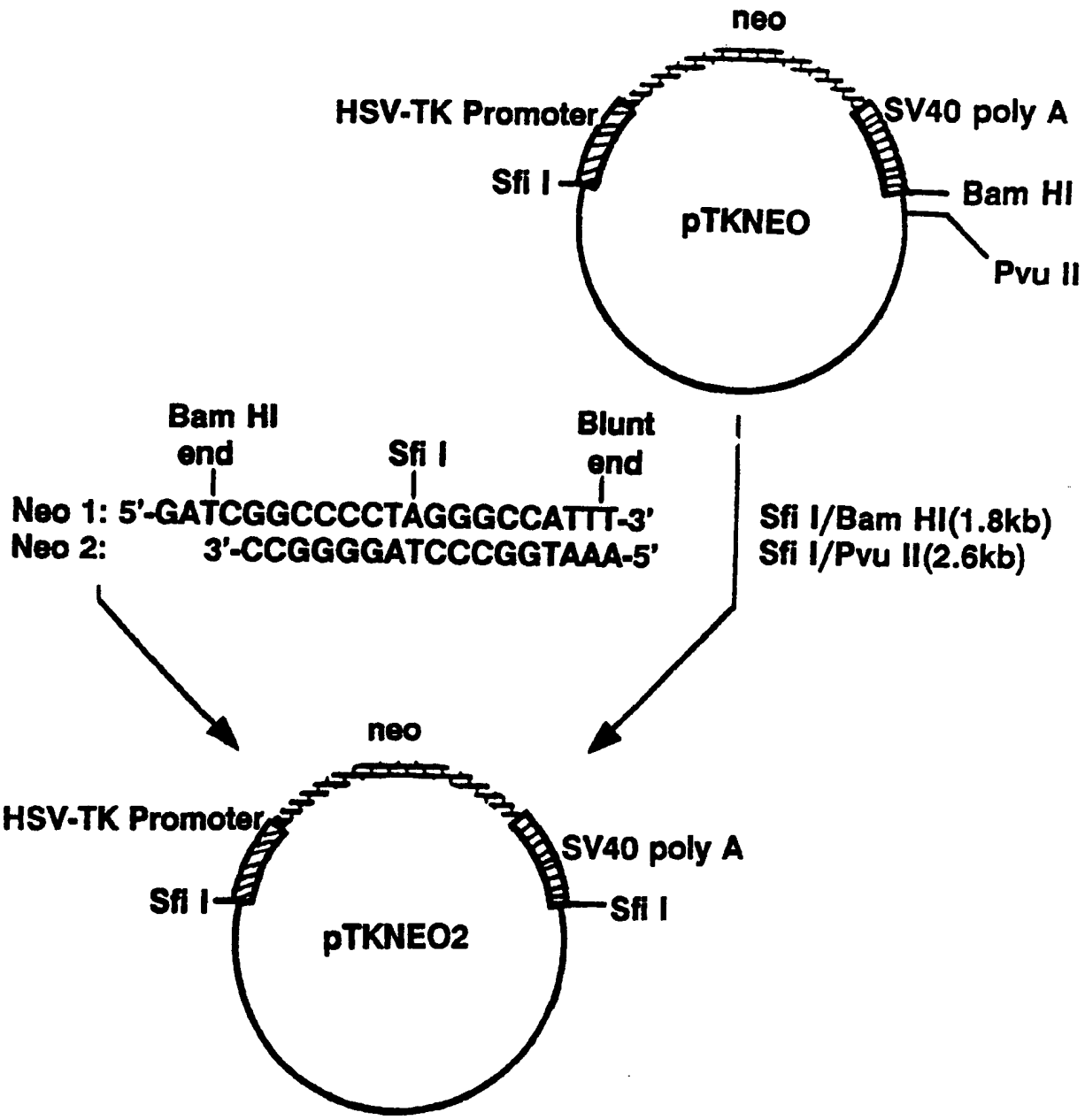


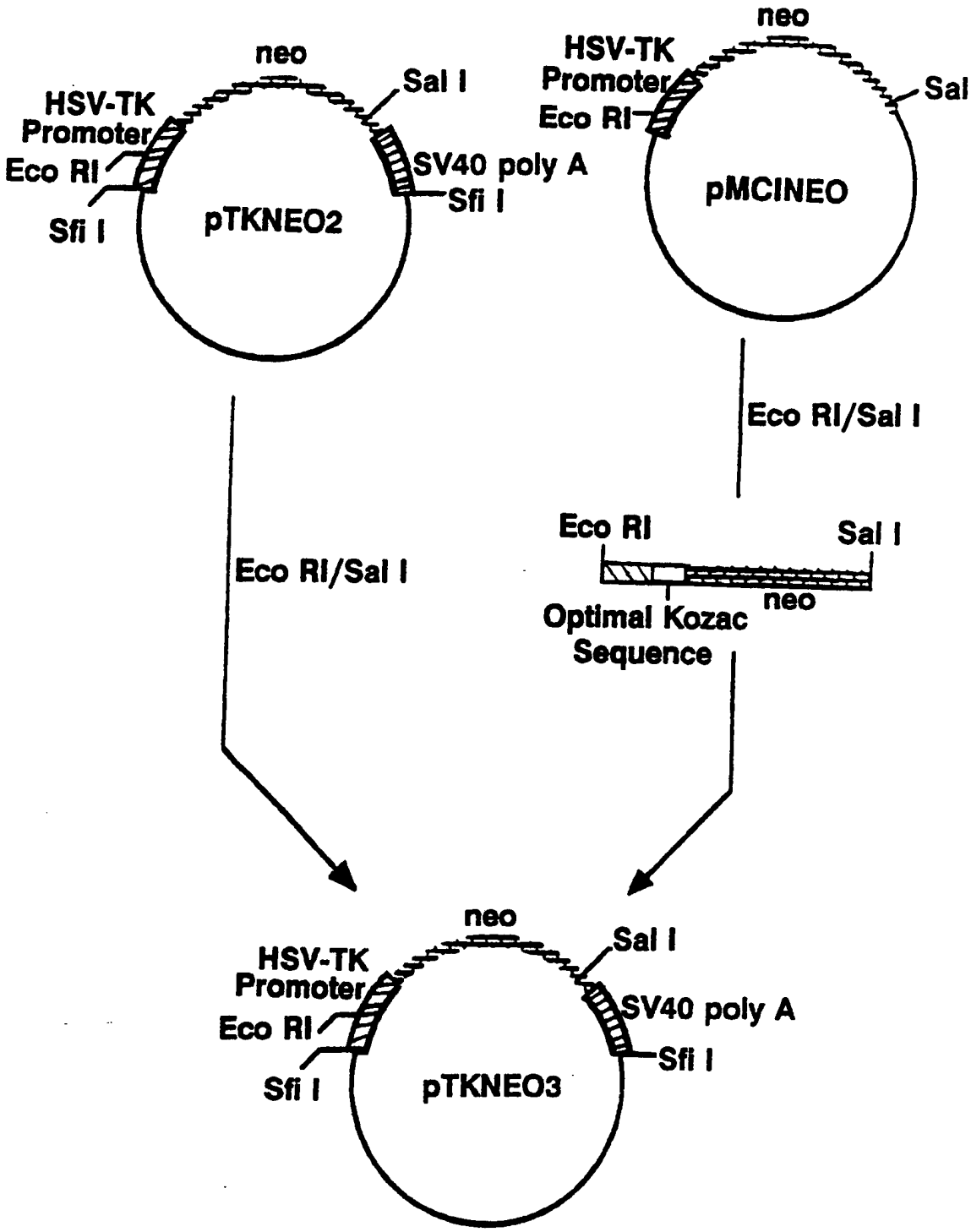
Figure 13

Construction of pTKNEO2



Figur 14

C nstruction of pTKNEO3



The Structure of pNEU106

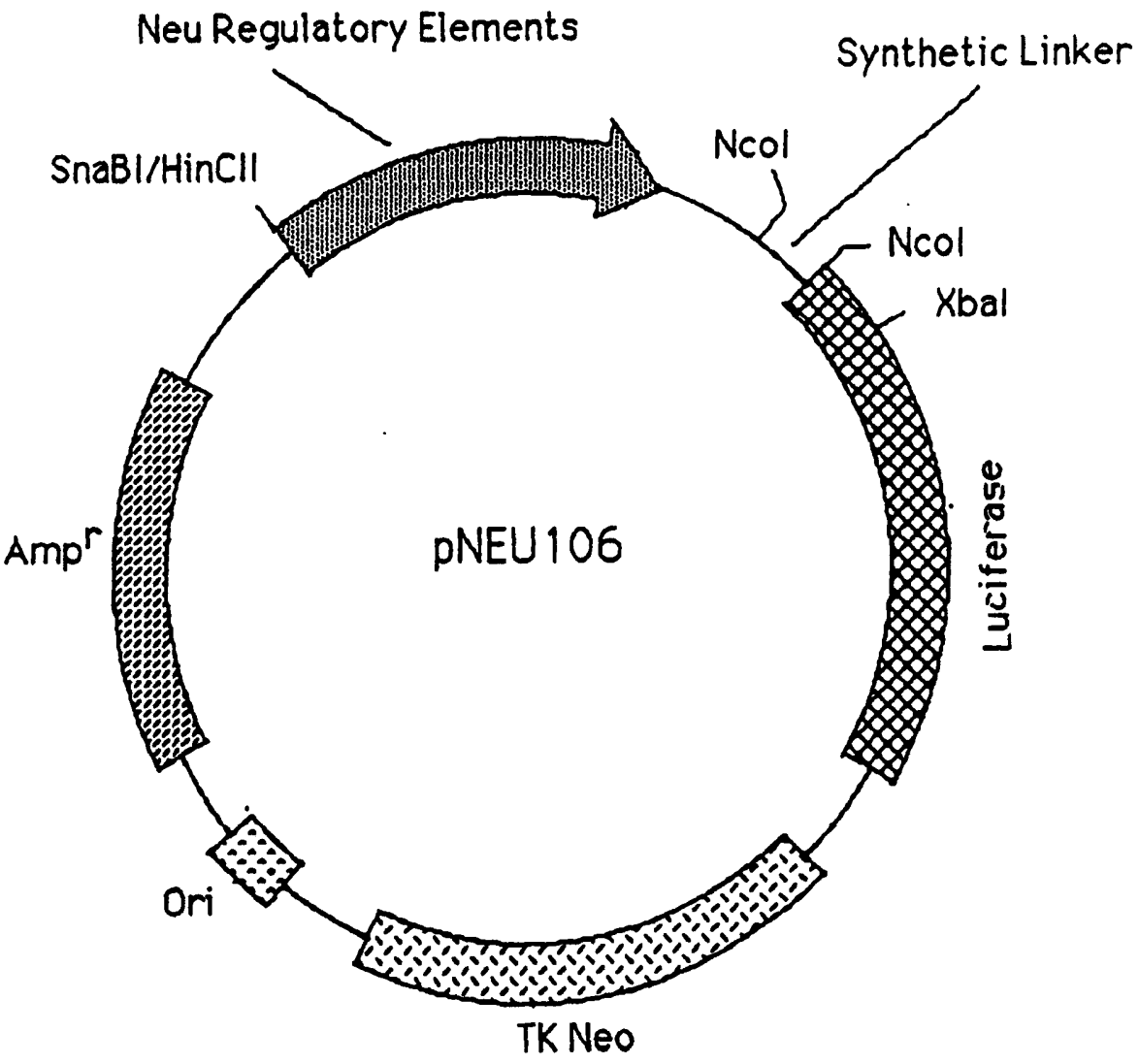


Figure16

The Structure of pKRAS106

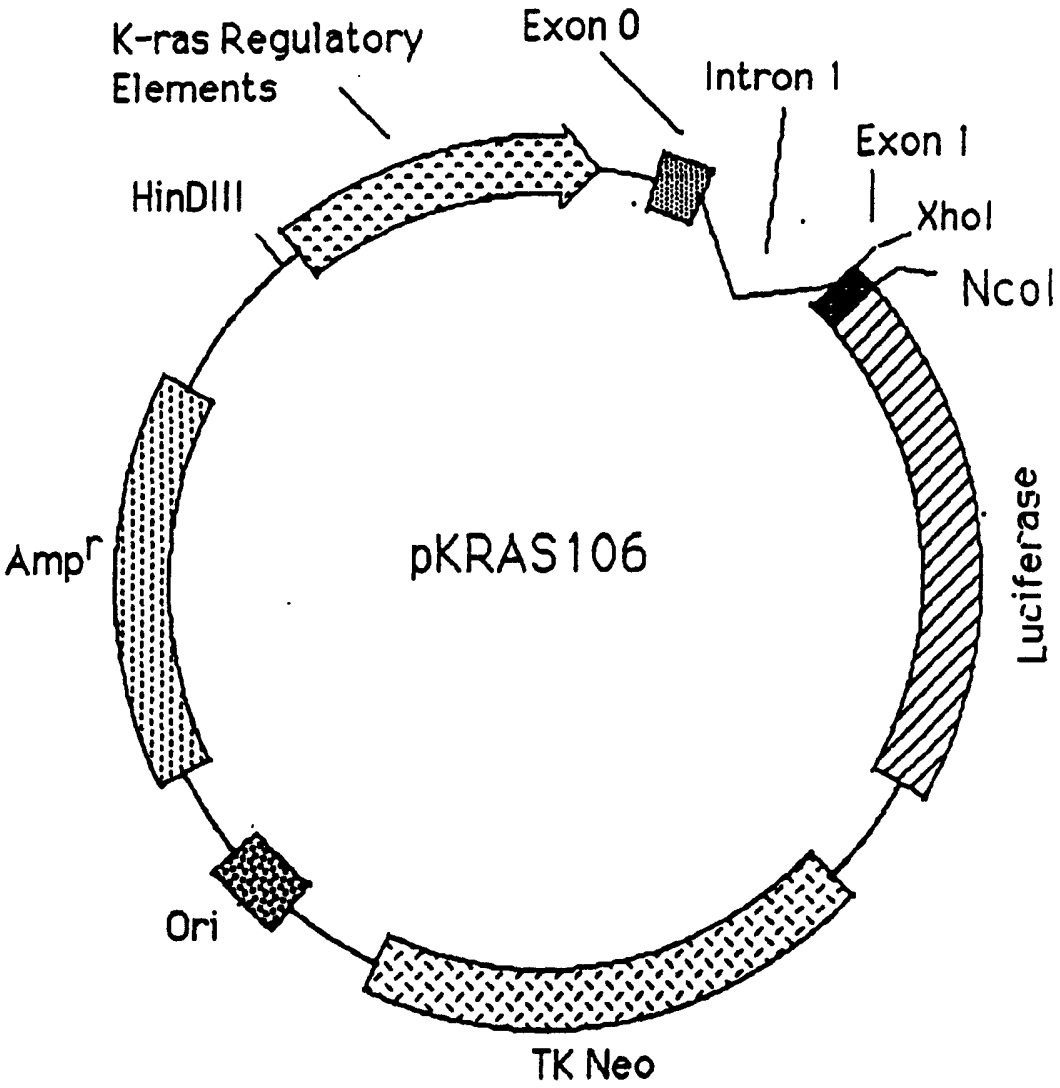
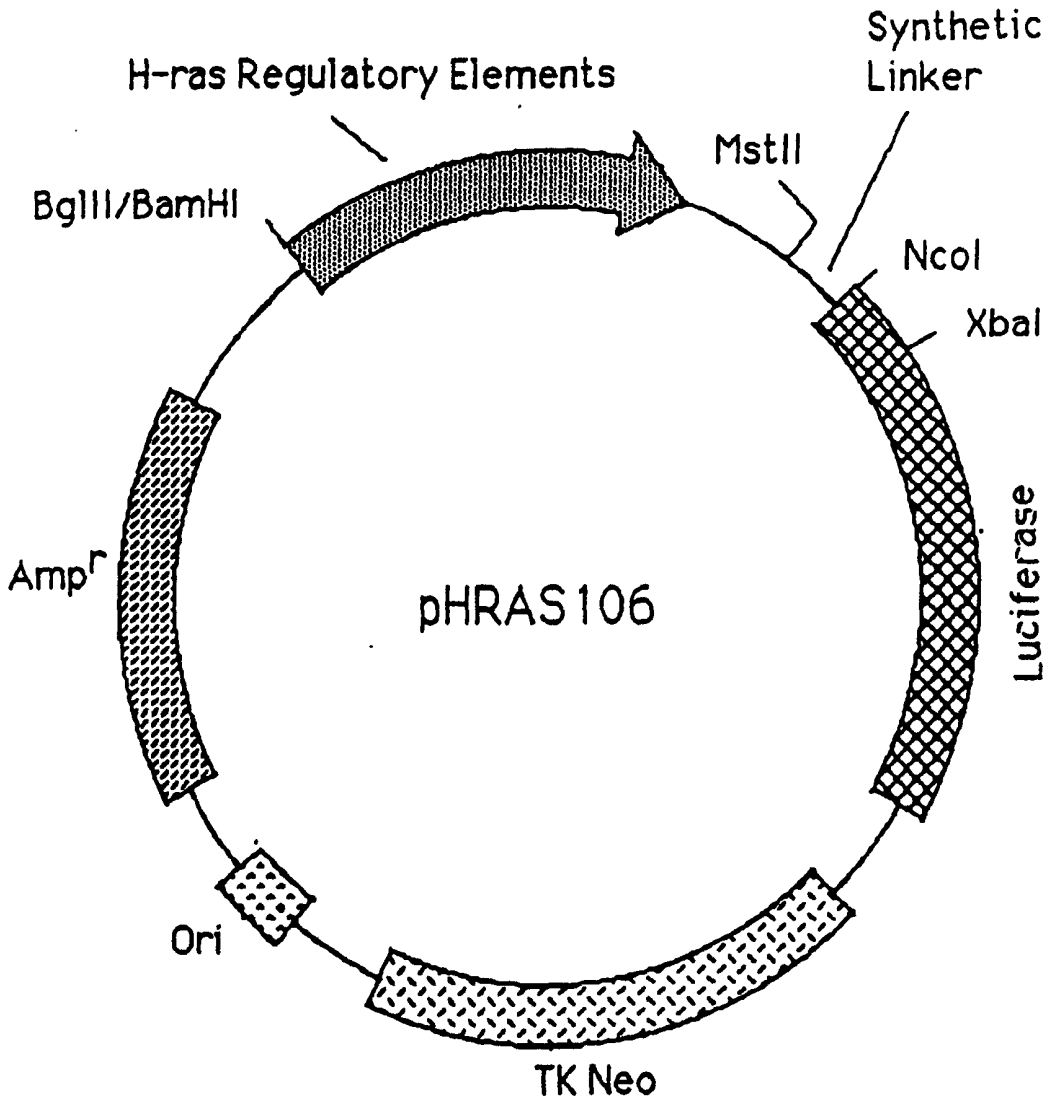


Figure 17

The Structure of pHRAS106



The Structure of
pNRAS106

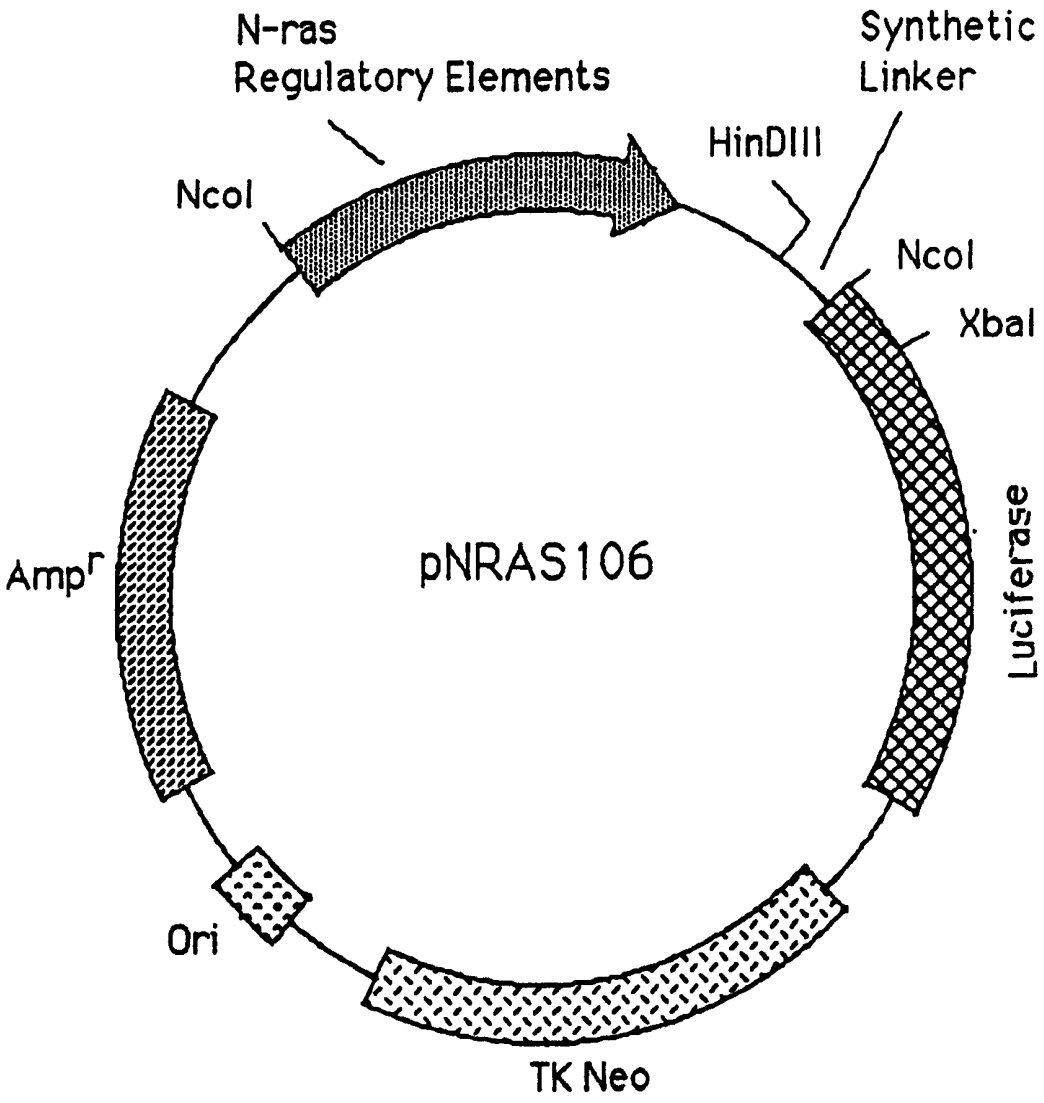
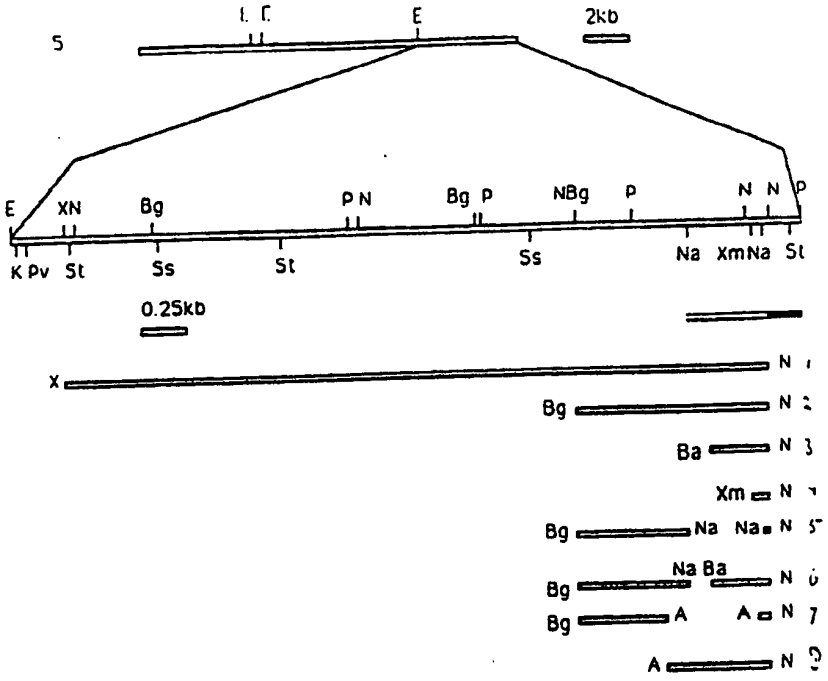


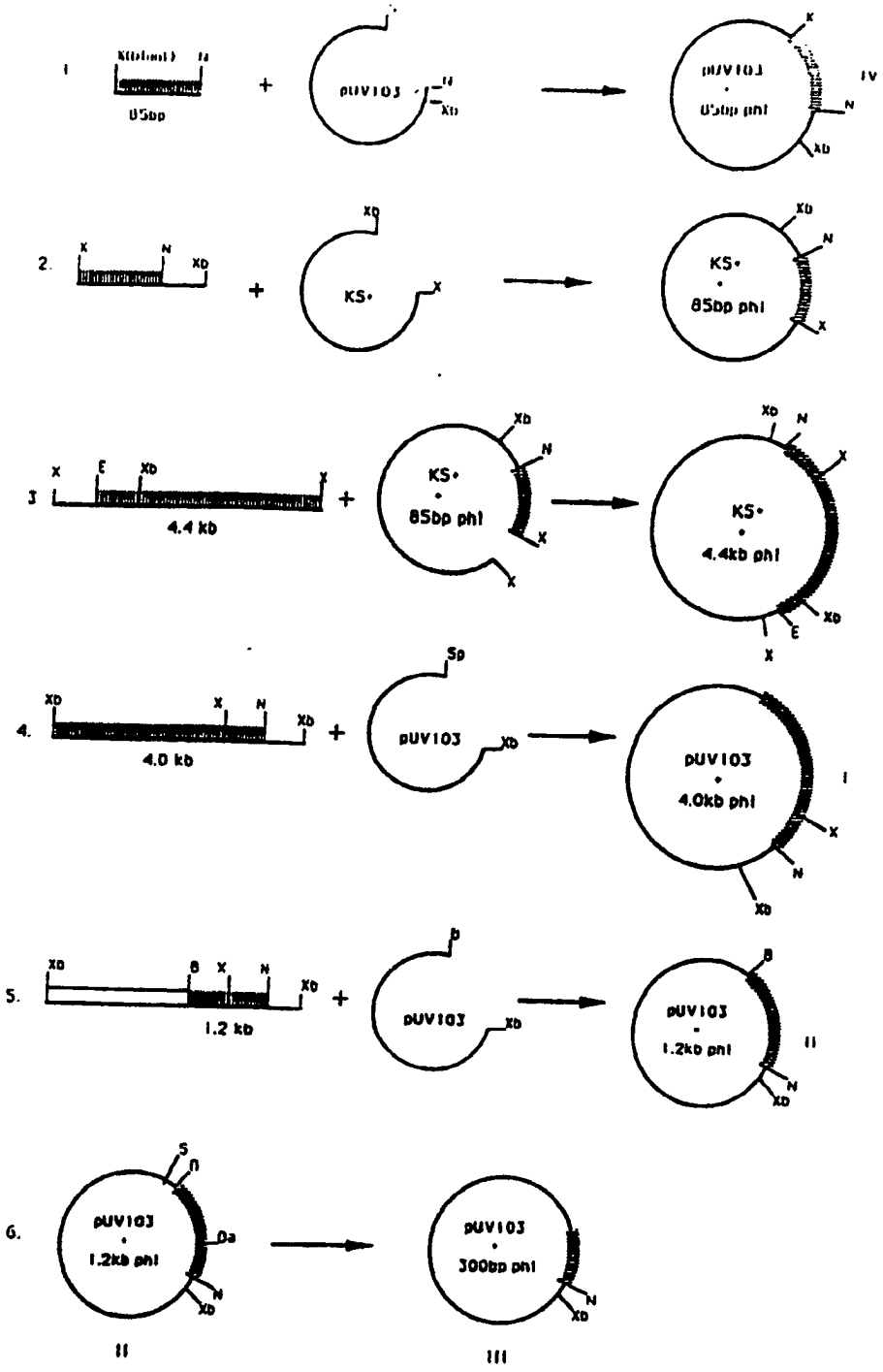
Figure 19



Organization of the phl promoter. Shown is an EcoRI map of 20kb of the phl promoter. Beneath this is a restriction enzyme map of the most 3' end containing the most 5' 175 bp of the coding region (from NcoI to PstI) of exon 1. The fragments numbered 1-8 have been cloned or are being cloned into the pUV103 vector.

E=EcoRI, K=KpnI, Pv=PvuII, X=XbaI, St=StuI, Bg=BglII, Ss=SstI, P=PstI, N=NcoI, Na=NaeI, Ba=BalI, A=ApaI. In the 4.4 kb EcoRI-PstI are no sites for BamHI, HindIII, EcoRV.

Figure 20



Cloning strategy of the construction of the phl promoter luciferase constructs I through IV. X=XmaI, N=NcoI, S=SnabI, Xb=XbaI, E=EcoRI, B=BglII and Ba=BalI.

FIGURE 21

The Structure of pP531106

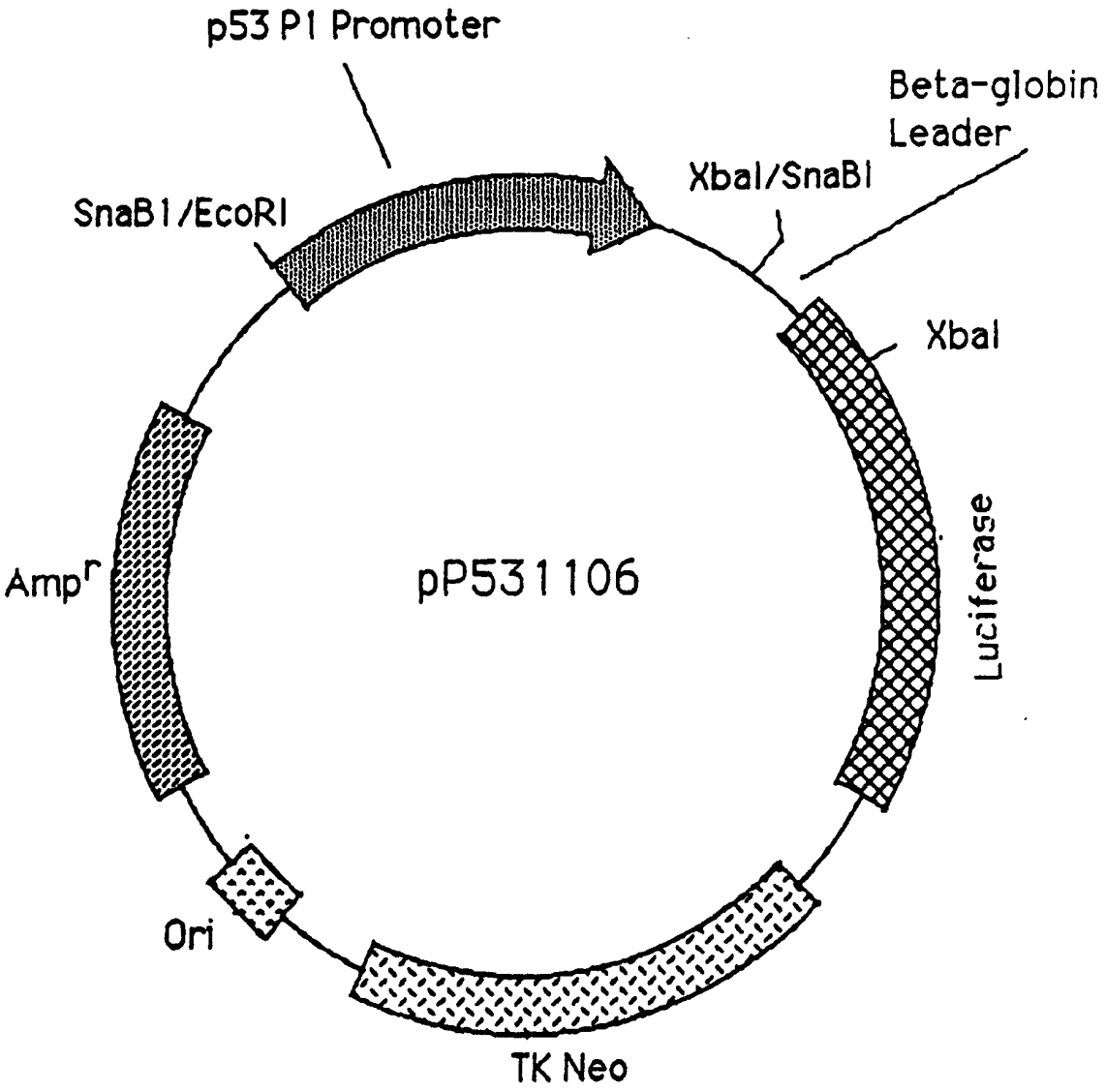


Figure 22

The Structure of pP532106

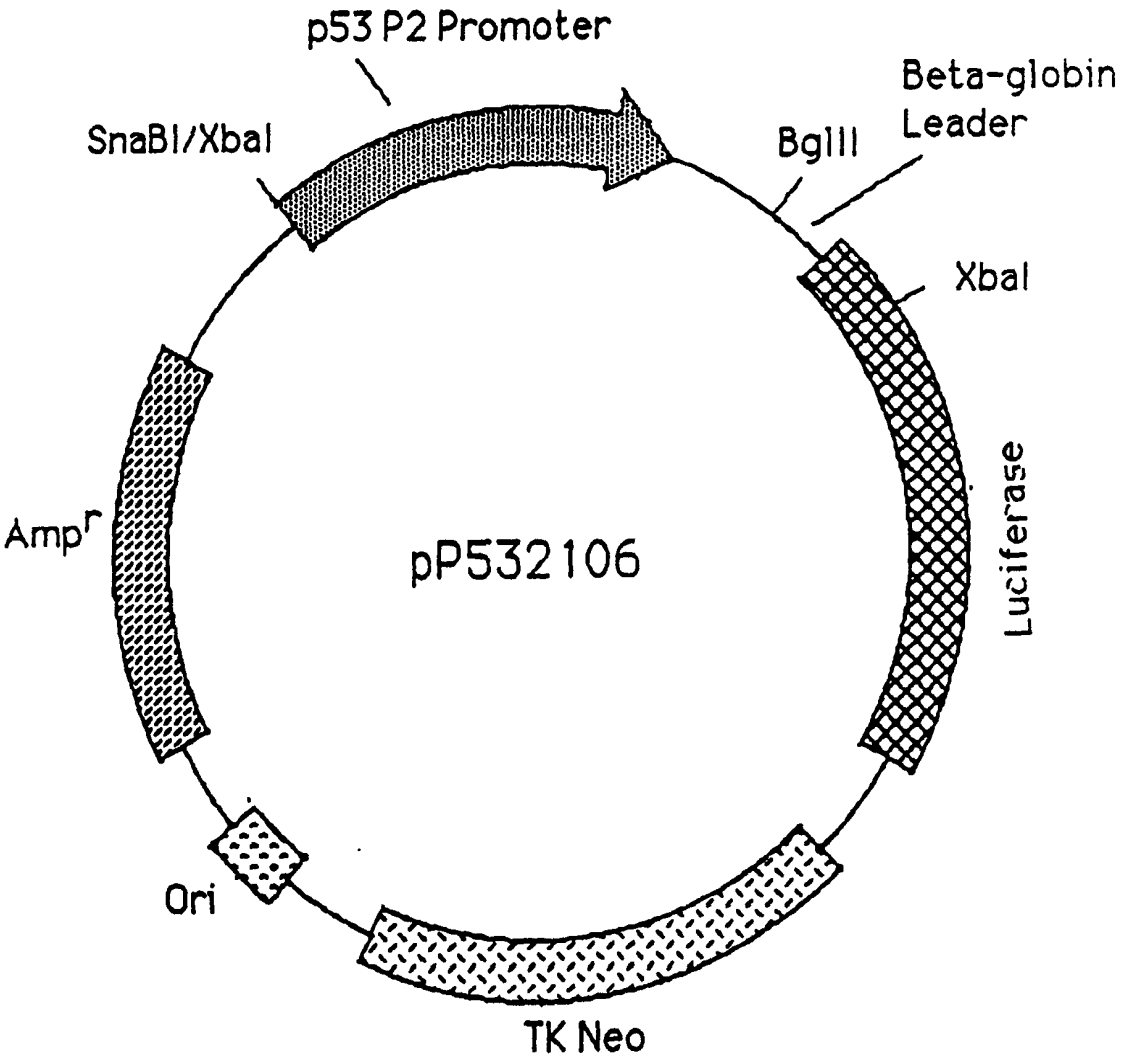


Figure 23

The Structure of pCM106

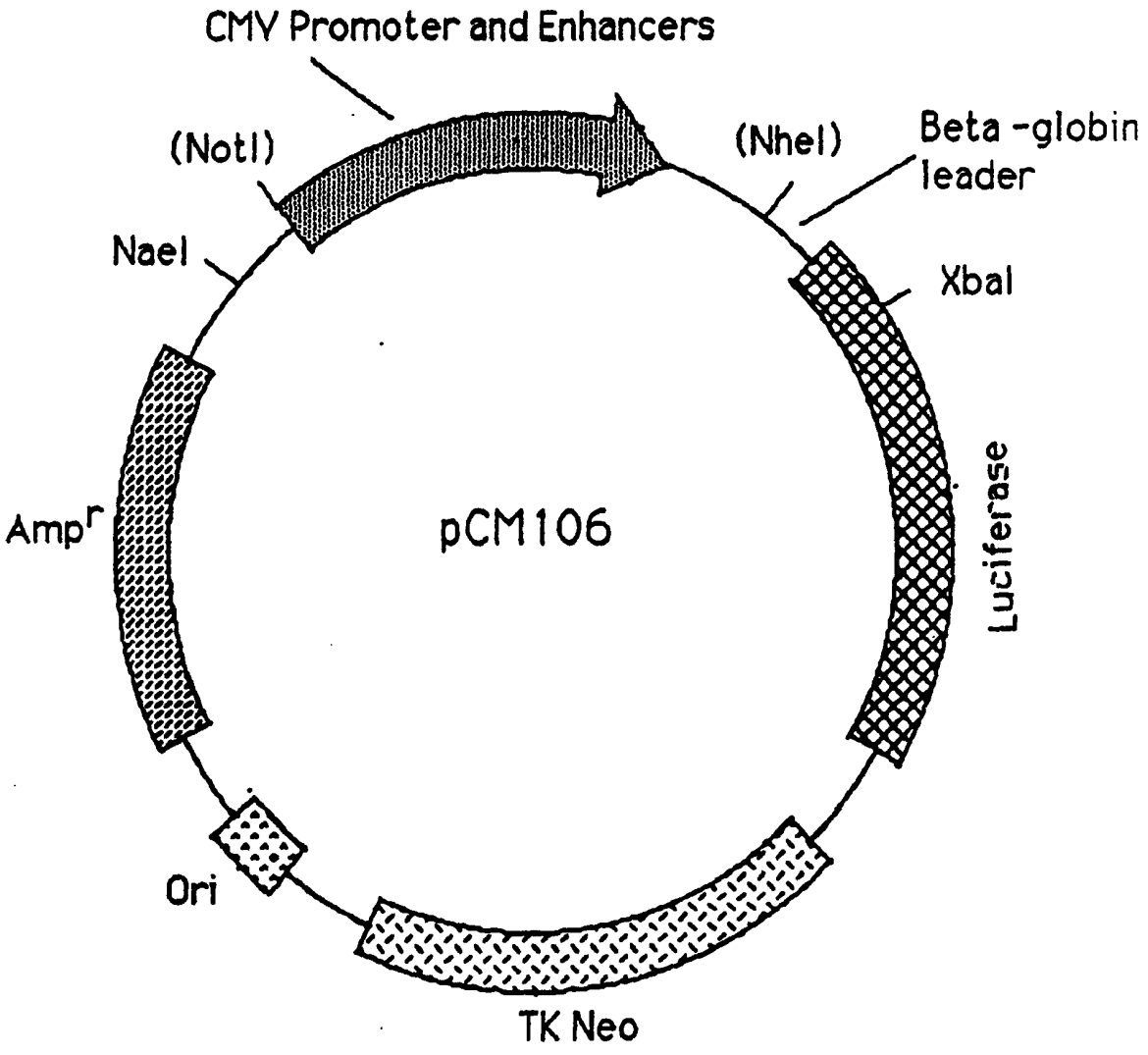


Figure 24

The Structure of
the neu Minigene

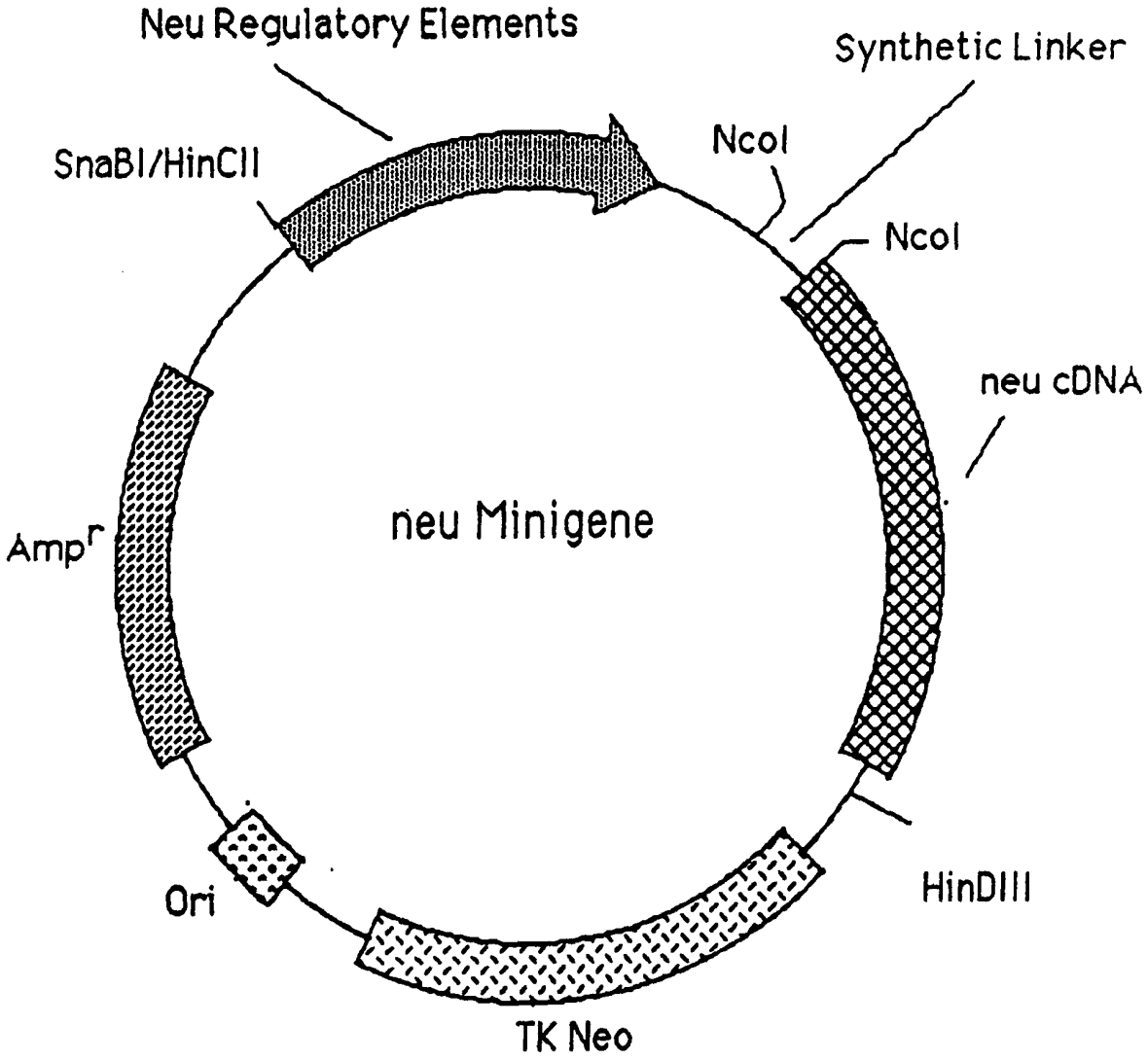


Figure 25

The Structure of the K-ras Minigene

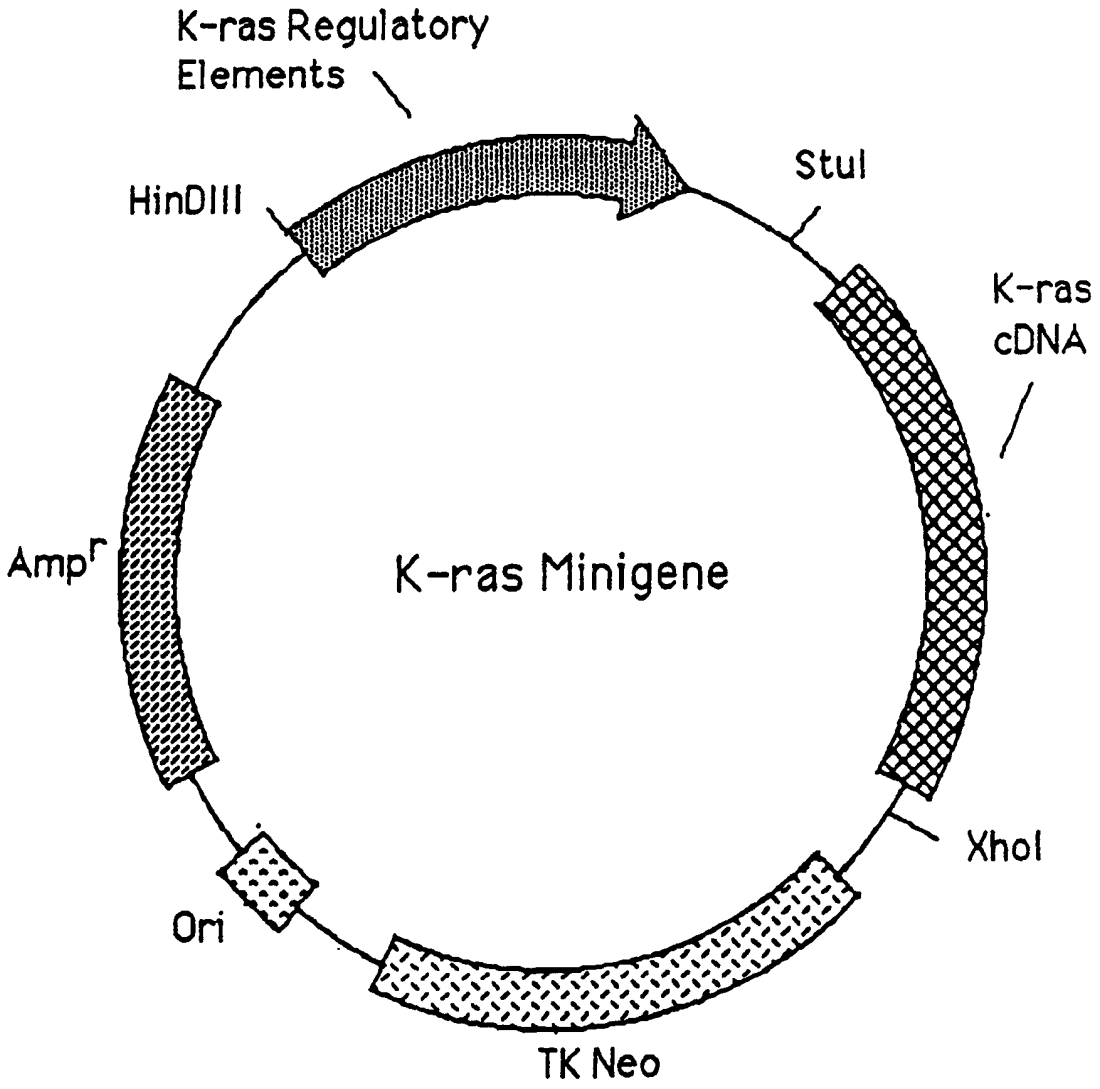
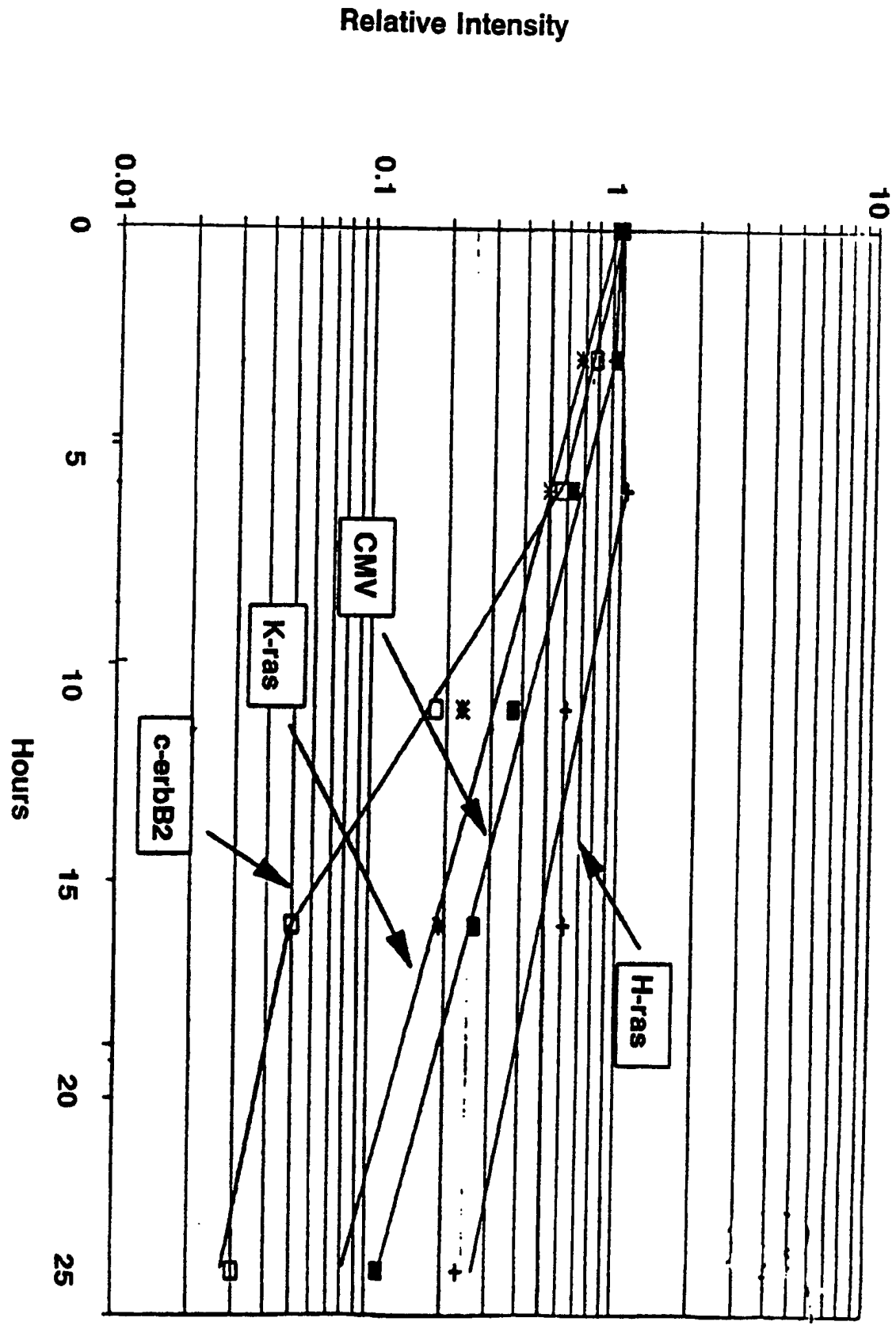
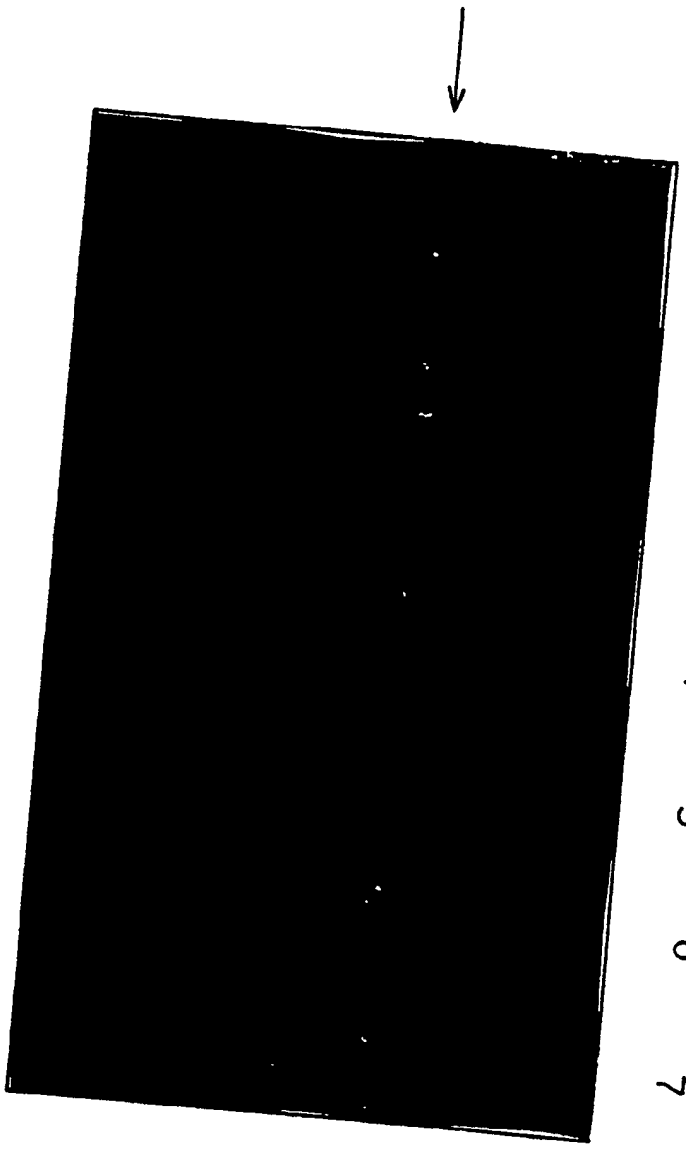


Figure 26


Relative Intensity
0.01
0.1
1
10
0
5
10
15
20
25
Hours
CMV
K-ras
c-erbB2
H-ras



Southern Blot
of Stable Clones

Figure 27

Figure 28
Quantitative PCR

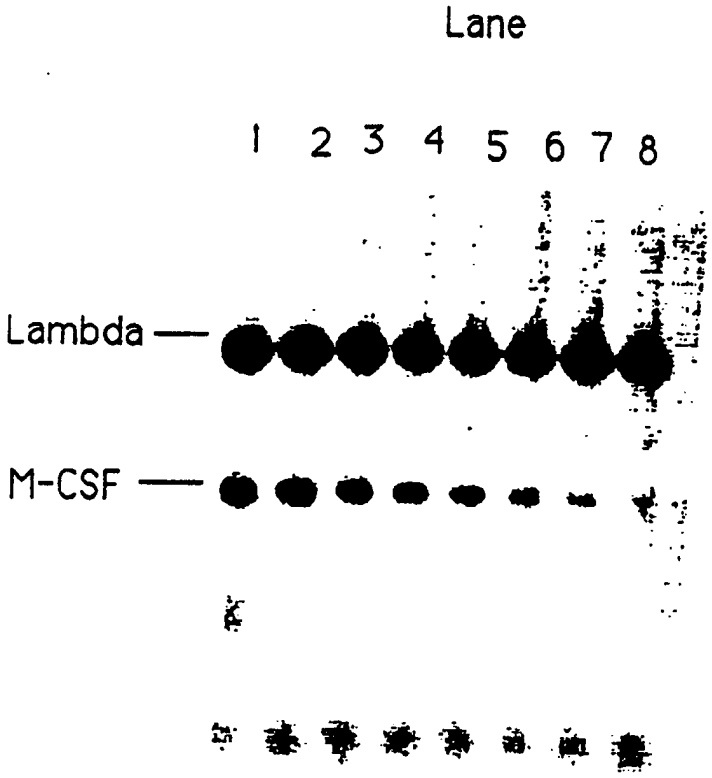


Figure 29

Quantitative PCR

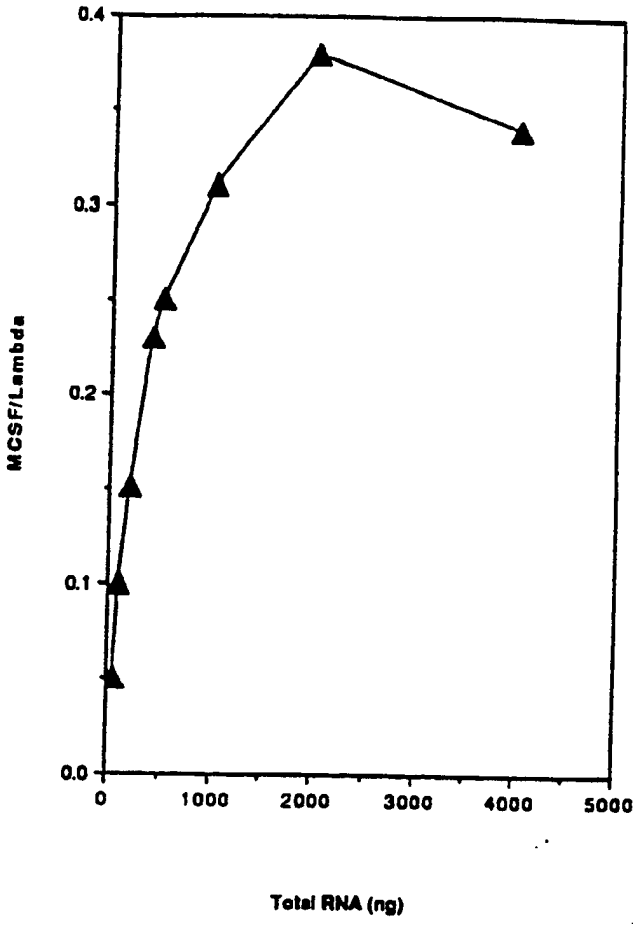


Figure 30
PCR of Oncogene mRNA

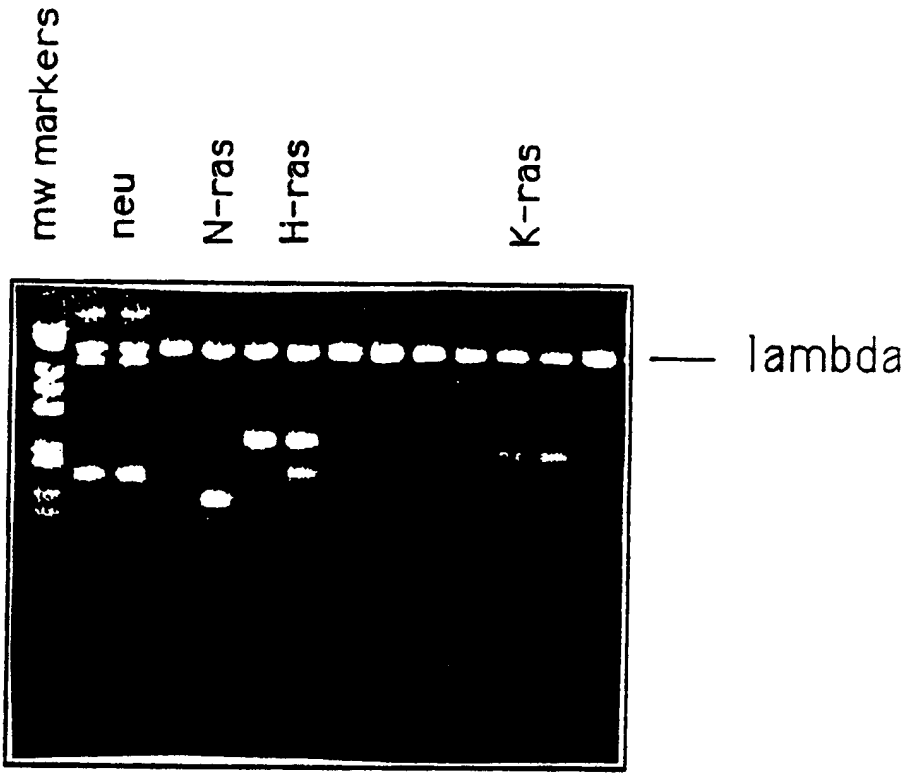


Figure 31

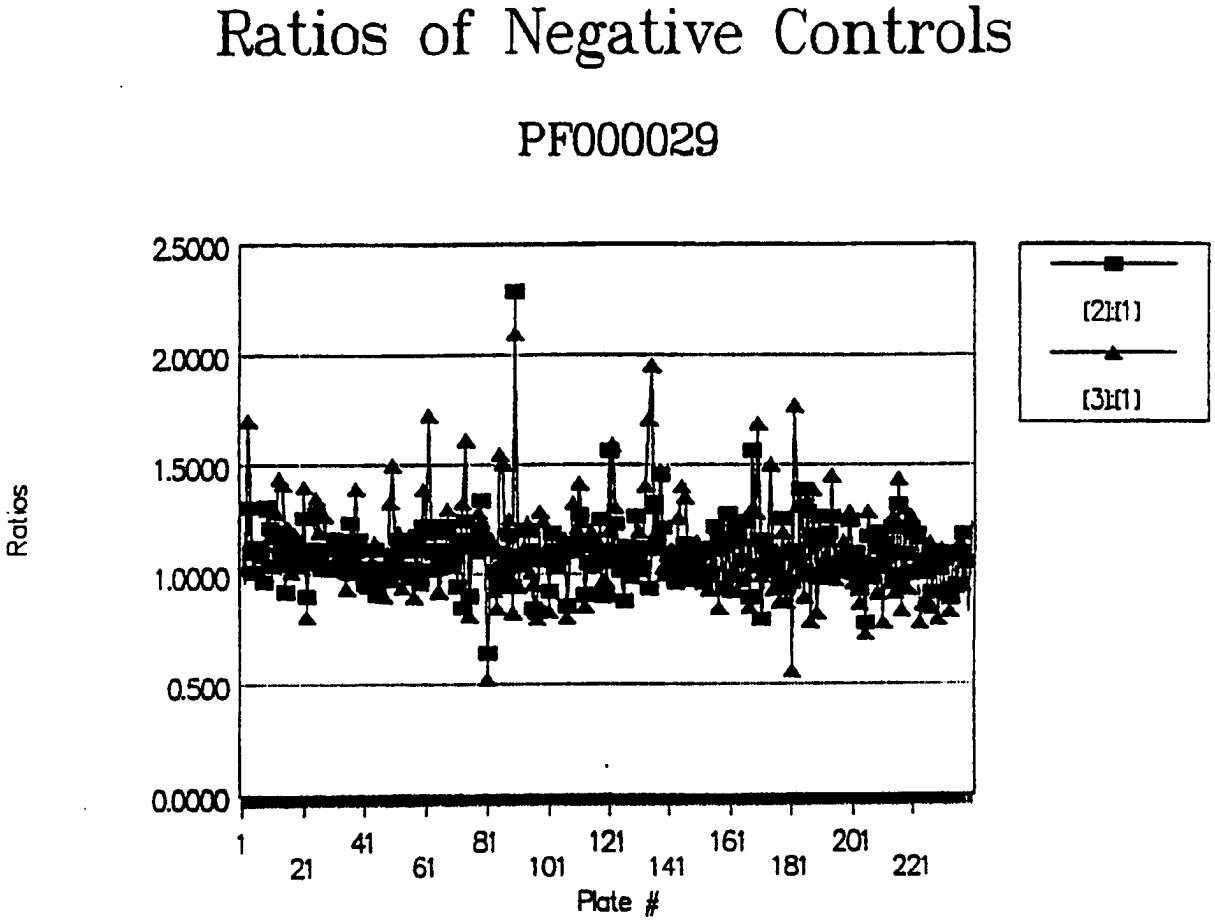

Ratios of Negative Controls
PF000029
Ratios
2.5000
2.0000
1.5000
1.0000
0.500
0.0000
1
21
41
61
81
101
121
141
161
181
201
221
Plate #
[2]H[1]
[3]H[1]

Figure 32

Average Negative RCOV

PF000029 - Avg.RCOV

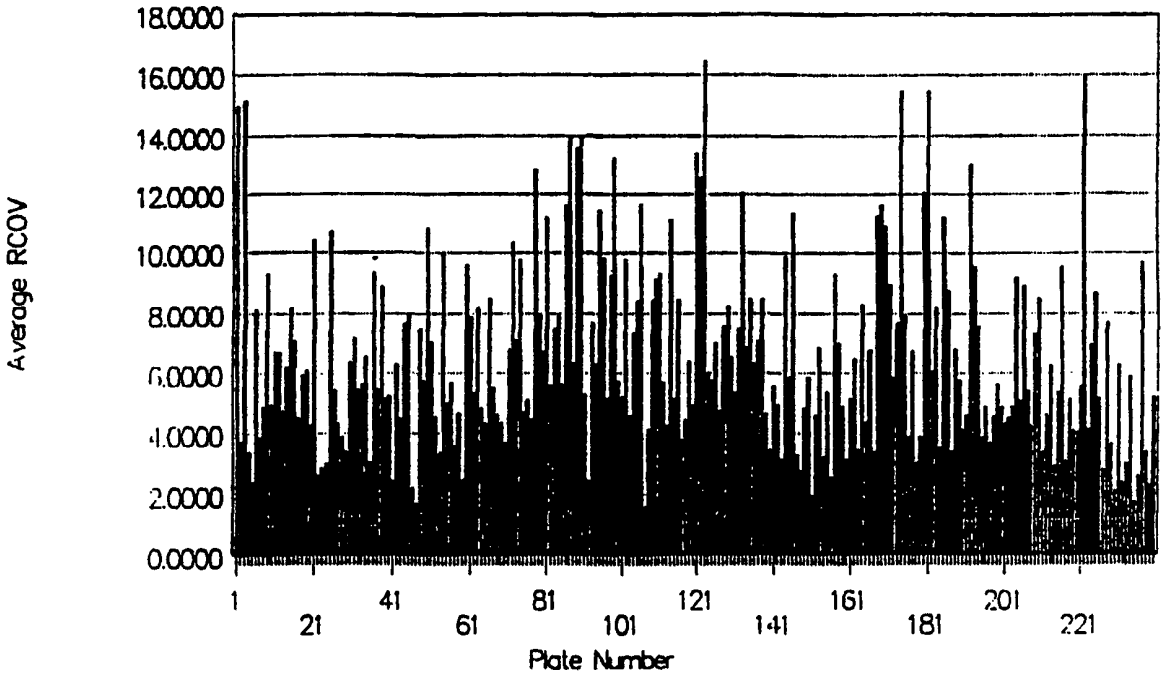


Figure 33

Average Positive Ctrl TIR RCOV

PF000029 + Avg.RCOV

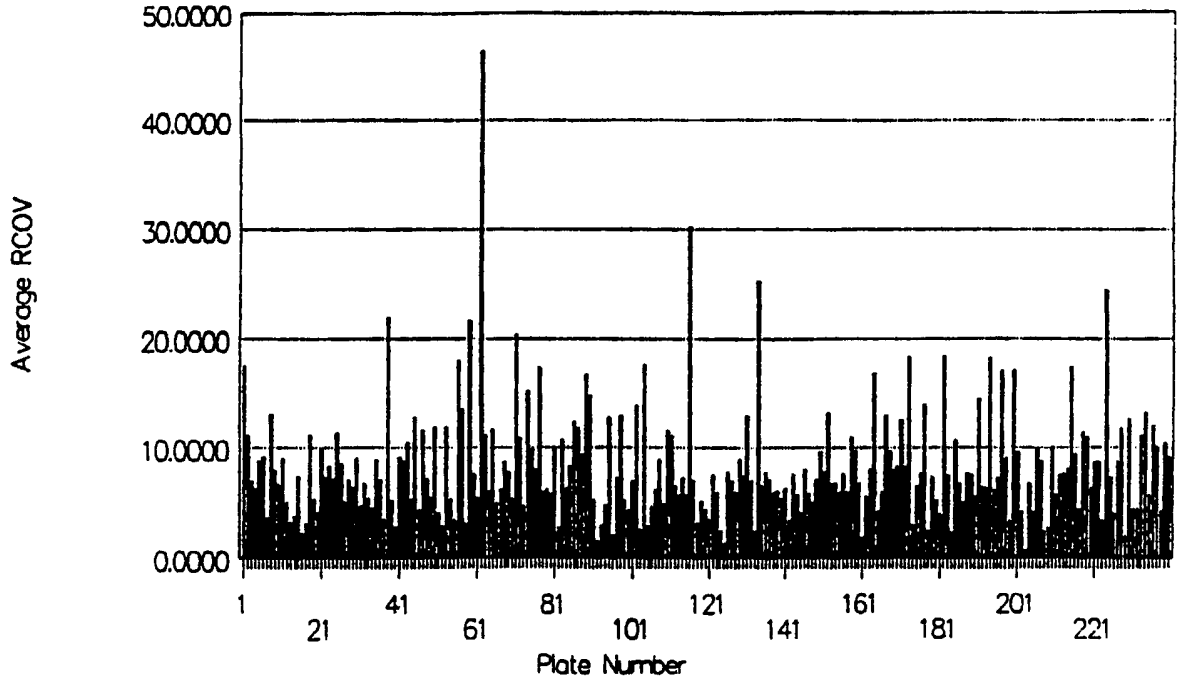
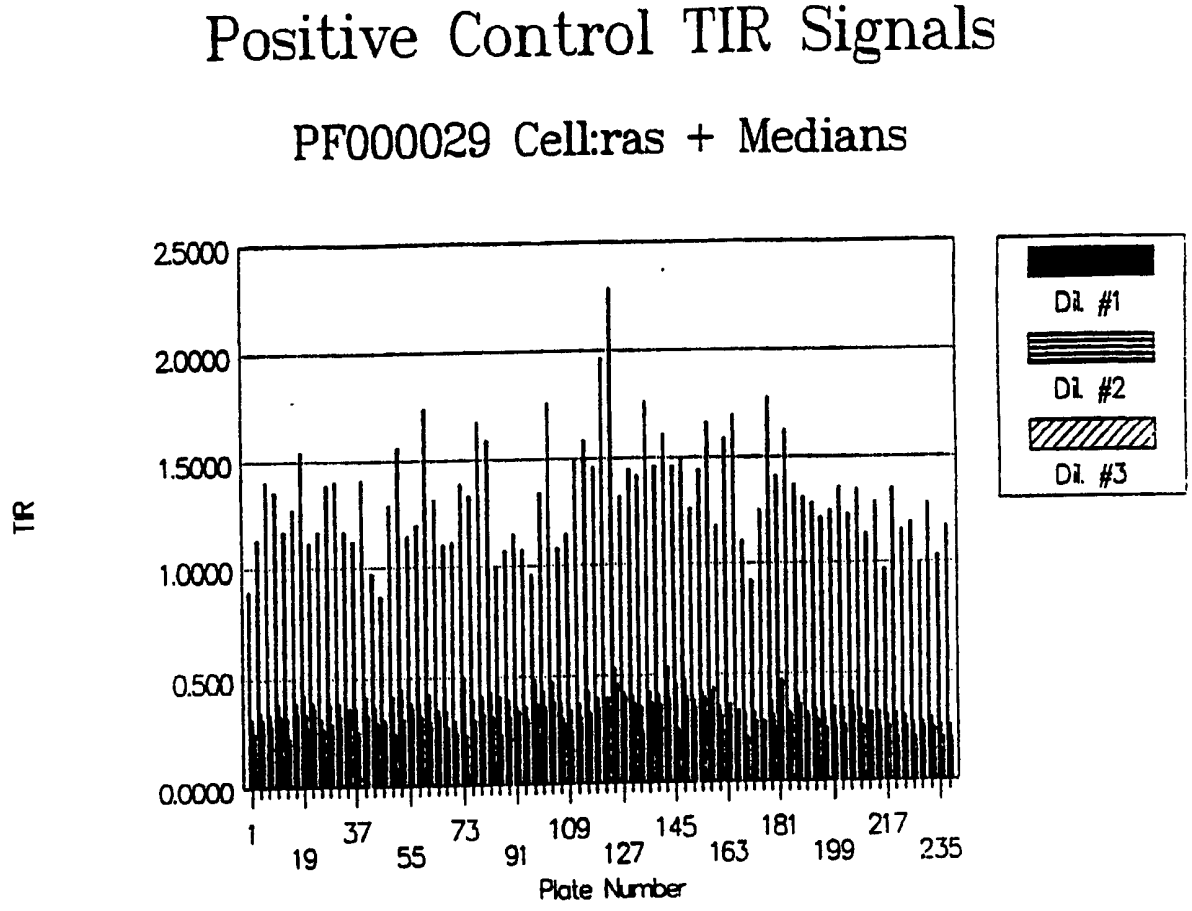


Figure 34


Positive Control TIR Signals
PF000029 Cell:ras + Medians
TIR
2.5000
2.0000
1.5000
1.0000
0.500
0.0000
1
19
37
55
73
91
109
127
145
163
181
199
217
235
Plate Number
Dil #1
Dil #2
Dil #3

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00421

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): Please See Attached Sheet.
US CL : 435/6, 7.1, 69.1, 70.1, 172.1, 172.2, 172.3, 236, 240.2, 244, 320.1

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/6, 7.1, 69.1, 70.1, 172.1, 172.2, 172.3, 236, 240.2, 244, 320.1

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[6]

Please See Attached Sheet.

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	US, A, 4,738,922 (Haseltine et al.) 19 April 1988. See col 1-10 and the examples.	1-23, 58-79
Y	US, A, 4,740,461 (Kaufman) 26 April 1988. See col 1-16 and the examples.	1-23, 58-79
Y	EP, A, 0,117,058 (Levinson et al., 29 August 1984. See at least the abstract and pages 5-18.	1-23, 58-79
Y	Mol. Cell. Biol. volume 7, no. 6, issued June 1987, Angel et al., "12-O-Tetradecanoyl-Phorbol-13-Acetate Induction Of The Human Collagenase Gene Is Mediated By An Inducible Enhancer Element Located In The 5'-Flanking Region", pages 2256-2266. See the abstract and the figures.	1-23, 58-79
Y	Proc. Natl. Acad. Sci. USA. Volume 83, issued May 1986 Kaushansky et al., "Genomic Cloning, Characterization, Multilineage Growth Promoting Activity Of Human Granulocyte-Macrophage Colony-Stimulating Factor", pages 3101-3105. See figure 2, and page 3105.	1-23, 58-79

* Special categories of cited documents:[16]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
05 MAY 1992	12 MAY 1992
International Searching Authority[1]	Signature of Authorized Officer[20]
ISA/US	Deborah Frieze for. Christopher Low

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

I. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C12P 21/00, 21/02; C12N 5/00, 7/04, 15/00, 1/38; C12Q 1/66, 1/68, 1/00

II. FIELDS SEARCHED
Other Documents Searched:

USPTO APS - USPAT, JPOABS
DIALOG - BIOSIS, CHINESE PATENT ABSTRACTS, CLAIMS/USPATENTS, INPADOC/FAMILY AND LEGAL STATUS, WORLD PATENT ABSTRACTS

Search Terms mammal, toxicity, toxic, chemical?, carcinogen?, oncogen?, transcript? breast, ovar?, panrea?, colon, lung, melanom? leukem?, tumor, suppress?

VI. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-32, 67-85, drawn to a method for effecting transcription of DNA coding for an oncogene or tumor suppressor , are classified in Class 435, subclasses 69.1 and 70.1.

II. Claims 33-34, 36-60, 62-66, drawn to a method of testing a compound for effecting transcription of DNA coding for an oncogene or tumor suppressor by measuring the amount of a polypeptide, are classified in Class 435, subclass 7.1.

III. Claims 35-50, 61-66, drawn to a method of testing a compound for effecting transcription of DNA coding for an oncogene or tumor suppressor by measuring the amount of mRNA produced, are classified in Class 435, subclass 6.

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	EMBO J. Volume 6, No. 9, issued September 1987, Ladner et al., "Human CSF-1: Gene Structure And Alternative Splicing Of mRNA precursors", pages 2693-2698. See at least pages 2693, 2696-2997.	1-23, 58-79
Y	EMBO J. Volume 6, No. 4, issued April 1987, Lefevre et al., "Tissue-Specific Expression Of The Human Growth Hormone Gene Is Conferred In Part By The Binding Of A Specific Trans-Acting Factor", pages 971-981. See at least page 971.	1-23, 58-79

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

Please See Attached Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. (Telephone Practice)

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	Proc. Natl. Acad. Sci. USA. Volume 82, issued November 1985, Lin et al., "Cloning And Expression Of The Human Erythropoietin Gene", pages 7580-7584. See entire document.	1-23, 58-79
Y	EMBO J. Volume 5, No. 3, issued March 1986, Nagata et al., "The Chromosomal Gene Structure And Two mRNAs For Human Granulocyte Colony-Stimulating Factor", pages 575-581. See at least the abstract.	1-23, 58-79
Y	Cell Volume 47, issued 10 October 1986, Yang et al., "Human IL-3 (Multi-CSF): Identification By Expression Cloning Of A Novel Hematopoietic Growth Factor Related To Murine IL-3", pages 3-10. See figure 4, and page 6.	1-23, 58-79
Y	de Serres et al., "Chemial Mutagens. Principles And Methods For Their Detection", published 1980 by Plenum Press (New York), pages 331, and 365-473. See at least pages 331, 367-369, 377.	1-23, 58-79
Y	Science Volume 227, issued 15 March 1985, Engebrecht et al., "Measuring Gene Expression With Light", pages 1345-1347. See entire document.	1-23, 58-79
Y	Science Volume 236, issued 05 June 1987, Maniatis et al., "Regulation Of Inducible Tissue-Specific Gene Expression", pages 1237-1245. See pages 1237, 1239, 1240, 1243.	1-23, 58-79
Y	Molec. Cell. Biol. Volume 7, No. 2, issued February 1987, de Wet et al., "Firefly Luciferase Gene: Structure And Expression In Mammalian Cells", pages 725-737. See the abstract, figures 1 and 3, pages 729-734.	1-23, 58-79
Y	Exp. Hematol., vol. 16, issued 1988, Bickel et al., "Granulocyte-Macrophage Colony-Stimulating Factor Regulation In Murine T Cells And Its Relation To Cyclosporin A", pages 691 695. See entire document.	1-23, 58-79
Y	US, A, 4,601,978 (Karin) 22 July 1986, see entire document.	1-23, 58-79
Y	WO, A, 89/02472 (Shannon et al.) 23 March 1989. See entire document.	1-23, 58-79
Y	Cell, vol. 49, issued 19 June 1987, Angel et al., "Phorbol Ester-Inducible Genes Contain A Common Cis Element Recognized By A TPA-Modulated Trans-Acting Factor", pages 729-739. See entire document.	1-23, 58-79
Y	Science, vol. 230, issued 18 October 1985, Kawasaki et al., "Molecular Cloning Of A Complementary DNA Encoding Human Macrophage-Specific Colony-Stimulating Factor (CSF-1)", pages 291-296, see entire document.	1-23, 58-79
Y	Proc. Natl. Acad. Sci. USA., vol. 81, issued August 1984, Kronke et al., "Cyclosporin A Inhibits T-Cell Growth Factor Gene Expression At The Level Of mRNA Transcription", pages 5214-5218. See entire document.	1-23, 58-79

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No.[18]
Y	Gene Amplification (Schimke, R. T., ed.), issued 1982, Mayo et al., "Altered Regulation Of The Mouse Metallothionein I Gene Following Gene Amplification Or Transfection", pages 67-73. See entire document.	1-23, 58-79
Y,P	US, A, 5,070,012 (Nolan et al.) 03 December 1991. See entire document.	24-51, 52-57
Y	US, A, 4,981,783 (Augenlicht) 01 January 1991 see entire document.	24-51, 52-57
Y	US, A, 4,806,463 (Goodchild et al.) 21 February 1989. See entire document.	24-41, 52-57
Y	US, A, 4,861,709 (Ulitzur et al.), 29 August 1989. See entire document.	24-41, 52-57
Y	US, A, 4,935,363 (Brown et al.), 19 June 1990. See entire document.	33-66
Y	Nature, vol. 346, issued 16 August 1990, Standaert et al., "Molecular Cloning And Overexpression Of The Human FK506-Binding Protein FKBP", pages 671-674. See entire document.	33-66
Y	J. Immunol., vol. 143, no. 2, issued 15 July 1989, Tocci et al., "The Immunosupprressant FK506 Selectively Inhibits Expression Of Early T Cell Activation Genes", pages 718-726. See entire document.	33-66
Y	Bio/Technol., vol. 7, issued March 1989, Ratner, "Can The Antisense Message Be Delivered?", page 207. See entire document.	33-66
Y	Bio/Techniques, vol. 7, no. 6, issued June 1989, Cao et al., "A Simple And Inexpensive System To Amplify DNA By PCR", pages 566-567. See entire document.	33-66
Y	Bio/Techniques, vol. 7, no. 6 issued June 1989, Lim et al., "A Simple Assay For DNA Transfection By Incubation Of The Cells In Culture Dishes With Substrates For Beta-Galactosidase", pages 576-579. See entire document.	33-66
Y	Bio/Technol., vol. 7, issued March 1989, McCall et al., "Biotherapy: A New Dimension In Cancer Treatment", pages 231-240. See entire document.	33-66
Y	Bio/Techniques, vol. 7, no. 10, issued November/December 1989, Brenner et al., "Message Amplification Phenotyping (MAPPing): A Technique To Simultaneously Measure Multiple mRNAs From Small Numbers Of Cells", pages 1096-1103. See entire document.	33-66
Y	US, A, 4,761,371 (Bell et al.) 02 August 1988. See entire document.	1-85
Y	Bio/Techniques, vol. 7, no. 10, issued November/December 1989, Munjaal et al., "In Situ Detection Of Progesterone Receptor mRNA In The Chicken Oviduct Using Probe-On Slides", pages 1104-1108. See entire document.	33-66

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No.[18]
Y	Bio/Techniques, vol. 7, no. 10, issued November/December 1989, Brasier et al., "Optimized Use Of The Firefly Luciferase Assay As A Reporter Gene In Mammalian Cell Lines", pages 1116-1122. See entire document.	33-66
Y	Bio/Techniques, vol. 7, no. 10, issued November/December 1989, Black et al., "Application Of The Multiscreen System To Cytokine Radioreceptor Assays", pages 1132-1138. See entire document.	33-66
Y	Bio/Techniques, vol. 8, no. 1, issued January 1990, Rao et al., "A Quantitative Assay For ß-D-Glucuronidase (GUS) Using Microtiter Plates", pages 38-40. See entire document.	33-66
Y	Bio/Techniques, vol. 8, no. 3, issued March 1990, Willingham et al., "A Reversible Multi-Well Chamber For Incubation Of Cultured Cells With Small Volumes: Application To Screening Of Hybridoma Fusions Using Immunofluorescence Microscopy", pages 320-324. See entire document.	33-66
Y	Bio/Techniques, vol. 9, no. 4, issued October 1990, Pons et al., "A New Cellular Model Of Response To Estrogens: A Bioluminescent Test To Characterize (Anti)Estrogen Molecules", pages 450-459. See entire document.	33-66
Y	US, A, 4,740,463 (Weinberg et al.) 26 April 1988. See entire document.	1-85
Y	US, A, 4,885,238 (Reddel et al.) 05 December 1989. See entire document.	1-85
Y	US, A, 4,699,877 (Cline et al.) 13 October 1987. See entire document.	33-66
Y	US, A, 4,736,866 (Leder et al.) 12 April 1988. See entire document.	1-85
Y	US, A, 4,535,058 (Weinberg et al) 13 August 1985. See entire document.	33-66